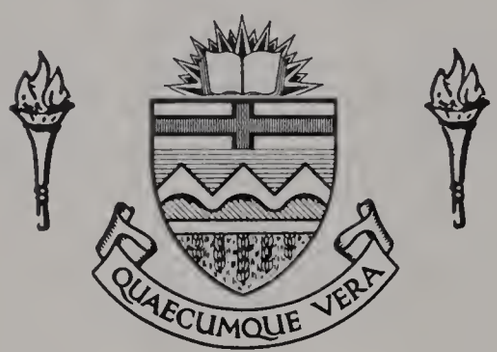

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ROTENONE-RESISTANT RESPIRATION IN MITOCHONDRIA ISOLATED FROM ETIOLATED PEA COTYLEDONS

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© ANNE M. JOHNSON-FLANAGAN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

"ROTENONE-RESISTANT RESPIRATION IN MITOCHONDRIA ISOLATED FROM ETIOLATED PEA COTYLEDONS"

submitted by Anne M. Johnson-Flanagan, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Mitochondria isolated from etiolated pea cotyledons by the sucrose density gradient technique exhibit resistance to rotenone while oxidizing NAD-linked substrates. The inhibited rate of α-ketoglutarate oxidation is equivalent to the recovered rate of malate oxidation (the recovered rate is the rate following the transient inhibition by rotenone). The inhibitory effect of rotenone on malate oxidation increases in response to increasing respiratory control ratios. The cyanide-resistant and rotenone-resistant pathways follow different courses of development and operation. The rotenone-resistant pathway transfers reducing equivalents to the cyanide-sensitive pathway. Malic enzyme was found to be competitively inhibited by rotenone concentrations as low as 1.67 μM. In pea cotyledon mitochondria rotenone is transformed into elliptone. This reduces its inhibitory effect on intact mitochondria. However, elliptone inhibits malic enzyme to the same extent as does rotenone. The products of malate oxidation reflect the interaction between malic enzyme and malate dehydrogenase. Rotenone inhibits the NADH dehydrogenase associated with malate dehydrogenase. Thus rotenone appears to exert its inhibitory effect on two enzymes of the electron transport chain of pea cotyledon mitochondria.

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LIST OF ABBREVIATIONS

ADP	-	adenosine 5' diphosphate
ADP:O	-	adenosine 5' diphosphate to oxygen ratio
ATP	-	adenosine 5'triphosphate
BSA	-	bovine serum albumin
cyt.	-	cytochrome
EDTA	-	ethylenediaminetetracetic acid
FP	-	flavoprotein
NAD	-	nicotinamide adenine dinucleotide
NADH	-	nicotinamide adenine dinucleotide (reduced)
NADP	-	nicotinamide adenine dinucleotide phosphate
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced)
n atom	-	nano atom
SHAM	-	salicylhydroxamic acid
Tes	-	N-tris(hydroxymethyl)-methyl-2-amino ethanesulphonic acid

INTRODUCTION

This investigation has been undertaken to study the rotenone-resistant pathway in plant mitochondria. The study of rotenone resistance has been the subject of much investigation for a number of years. During this time, many different ideas have emerged in an attempt to explain the ability of plant mitochondria to continue to respire in the presence of rotenone. These have not been totally substantiated and in fact, many have been refuted.

Palmer in a recent review (1976), questions the biochemical benefit to be derived from density gradient purification of mitochodria. Many workers have used mitochondria prepared by simple differential centrifugation in their studies of rotenone resistance. The validity of this procedure is investigated in this thesis.

Another practice that could lead to erroneous results is the seemingly indiscriminate use of inhibitors and cofactors to activate and stimulate the rotenone-resistant pathway. The present investigation of the rotenone-resistant pathway has been done using only rotenone as the activator of the pathway.

Aspects of this study include the relationship of the rotenone-resistant pathway to the cyanide-resistant pathway, substrate and cofactor effects and the mechanism for the deactivation of rotenone. The enzymes responsible for the oxidation of malate are also studied. On the basis of the present investigation a possible explanation for the control and operation of the rotenone-resistant pathway is developed.

LITERATURE REVIEW

Preparative Methods. In early work mitochondria were prepared by simple differential centrifugation. Studies of mitochondrial intactness and purity have shown that this method is not always adequate (James and Spencer, 1979; Solomos, Malhotra, Prasad and Spencer, 1972). Microsomal contamination, lack of homogeneity and the presence of cytochrome b containing organelles are a few of the problems encountered when this method is employed (Douce, Manella and Bonner, 1973; James and Spencer, 1979; Solomos et al, 1972). Homogeneity and minimal contamination can be achieved through the use of density gradients (Douce et al, 1972). Despite this, many workers continue to prepare mitochondria by simple differential centrifugation. In studies that involve inhibitors, the presence of microsomes could increase the inhibited respiration rate as well as decrease the uptake of the inhibitor into the mitochondria. The inhibitory effect of rotenone is inversely proportional to the protein concentration (Ernster, Dallner and Azzone, 1963; Oberg, 1961) thus any increase in extra mitochondrial protein would decrease the effect on the mitochondria.

Rotenone Resistance. For a number of years rotenone has been used in studies of the electron transport chain of mitochondria. In the past decade the presence of a rotenone-resistant pathway was recognized in a number of yeasts and higher plants. Unlike plants, animal mitochondria lack this pathway and consequently oxidation of all NAD-linked substrates is inhibited by rotenone (Gutman, Singer and Casida, 1970; Ikuma and Bonner, 1967; Oberg, 1961). The site

of inhibition is one of nonhaem iron centres associated with the internal NADH dehydrogenase complex (Oberg, 1961; Ohnishi, 1973; Ragan and Garland, 1971; Teeter, Baginsky and Hatefi, 1969; Wilson and Hanson, 1969). Very low concentrations (1-2 μM) are sufficient to cause this inhibition (Gutman et al, 1970; Oberg, 1961). Higher rotenone concentrations inhibit more than one site of the respiratory chain (Teeter et al, 1969), one of these being cytochrome b (Yamamoto, Unai, Ohkawa and Casida, 1971).

The Exogenous NADH Dehydrogenases. The ability to oxidize exogenous NADH is a basic difference between plant and animal mitochondria (Cunningham, 1964; Ikuma, 1972; Palmer and Coleman, 1974). Initially, the ability of plant mitochondria to oxidize exogenous NADH was thought to be caused by poor isolation techniques (Lieberman and Baker, 1965). It was expected that NADH was being oxidized by the internal NADH dehydrogenase because of leaky mitochondria (Ikuma, 1972; Lieberman and Baker, 1965). Subsequently, rapid oxidation of exogenous NADH was found in apparently intact yeast mitochondria (Ohnishi, Kawagushi and Hagihara, 1966). As the inner mitochondrial membrane is impermeable to NAD and NADH in yeast, the internal NADH dehydrogenase could not be responsible (Von Jagow and Klingenberg, 1976). Cunningham (1964) was the first to postulate that exogenous NADH is oxidized by an external NADH dehydrogenase. The dehydrogenase bypasses the flavoprotein responsible for the oxidation of endogenous NADH and is therefore coupled to only two sites of ATP synthesis (Coleman and Palmer, 1971; Cunningham, 1964; Day and Wiskich, 1974a; Douce et al 1973;

Ikuma and Bonner, 1967; Moreau, 1976; Palmer and Coleman, 1974; Palmer and Passam, 1970).

This external NADH dehydrogenase is on the outside of the inner membrane (Douce et al, 1973; Marx and Brinkmann, 1978; Palmer and Passam, 1970). Exogenous cytochrome c is not required for the transfer of reducing equivalents (Coleman and Palmer, 1972). Divalent cations are required for the operation of this dehydrogenase (Coleman and Palmer, 1971; Palmer and Coleman, 1974). Inhibitor studies of this dehydrogenase have shown it to be insensitive to rotenone and strongly inhibited by antimycin A, indicating that reducing equivalents enter the electron transport chain on the substrate side of cytochrome b and bypass site one (Coleman and Palmer, 1972; Day and Wiskich, 1974a; Moreau and Lance, 1972). The pathway may involve one of the flavoproteins detected by Storey and Bahr (1972).

Ferricyanide, an electron acceptor that cannot penetrate the inner mitochondrial membrane, has been used to study this dehydrogenase. However, ferricyanide will react with many flavoproteins, including the NADH cytochrome b_{555} reductase of the outer membrane (Douce et al, 1973; Marx and Brinkmann, 1978). Recent work has suggested an inability of ferricyanide to react with the external NADH dehydrogenase unless the mitochondria are damaged (Douce et al, 1973). Therefore, the ferricyanide cannot provide conclusive proof for the existence of an external NADH dehydrogenase linked directly with the respiratory chain (Palmer and Coleman, 1974). There is evidence for and against the involvement of this dehydrogenase in the rotenone-resistant pathway.

The other external NADH dehydrogenase that oxidizes exogenous NADH is located on the outer mitochondrial membrane (Day, Rayner and Wiskich, 1976; Douce et al, 1973; Moreau and Lance, 1972). It is associated with flavoprotein and cytochrome b_{555} (Day and Wiskich, 1975; Douce et al, 1973; Moreau, 1976; Moreau and Lance, 1972). Oxidation of NADH via the dehydrogenase is coupled to one site of ATP synthesis (Moreau, 1978). It is not inhibited by rotenone or antimycin-A (Day and Wiskich, 1974a; Douce et al, 1973; Moreau and Lance, 1972).

This pathway is found in almost all isolated plant mitochondria (Day and Wiskich, 1974a; Douce et al, 1973; Moreau, 1976; Moreau and Lance, 1972; Palmer and Passam, 1976; Wilson and Hanson, 1967). A requirement for the activity of this pathway is a supply of exogenous cytochrome c (Day et al, 1976; Douce et al, 1973; Moreau, 1976; Moreau, 1978; Moreau and Lance, 1972). Cytochrome c may be reduced by the NADH dehydrogenase and then cross the intermembrane space and be oxidized by cytochrome oxidase. This oxidation would result in the production of ATP (Moreau, 1978; Palmer and Coleman, 1974). This pathway is not expected to be involved in the rotenone-resistant pathway of NAD-linked substrates (Wiskich and Day, 1979).

The NADH dehydrogenase of the inner membrane can be distinguished from the NADH dehydrogenase on the outer membrane by their requirement for different stereoisomers of NADH. The NADH dehydrogenase on the outer membrane is α-specific and the NADH dehydrogenase on the outside of the inner membrane is β-specific (Sottocasa, Kuylenstierma, Ernster and Bergstrand, 1967). The NADH dehydrogenase on the outside of the inner membrane has been demonstrated to be β-specific in

yeast (Von Jagow and Klingenberg, 1970). There is strong evidence that this is also the case in higher plants (Douce et al, 1973).

These two pathways for the oxidation of exogenous NADH may be able to interact. Antimycin-A inhibited NADH oxidation can be relieved by the addition of cytochrome c (Day and Wiskich, 1974a; Douce, Christensen and Bonner, 1973).

The Internal NADH Dehydrogenases. Current work suggests that there are two internal NADH dehydrogenases present in the inner mitochondrial membrane (Brunton and Palmer, 1973; Day and Wiskich, 1974a; Palmer and Arron, 1976; Wilson and Hanson, 1969). These both are approachable only from the matrix side and oxidize endogenous NADH only. The rotneone-sensitive dehydrogenase is coupled to three sites of phosphorylation, whereas the rotenone-resistant dehydrogenase is coupled to two sites of phosphorylation (Palmer, 1976).

The rotenone-sensitive dehydrogenase is a flavoprotein having flavin mononucleotide (FMN) as the prosthetic group (Rao, Felton, Heunnekens and Mackler, 1963). It also contains at least four iron sulphur centres. The site of rotenone inhibition is expected to be FeS_2, the last iron sulphur centre to be reduced. Piericidin A also inhibits at this site (Palmer, 1976).

This pathway of NADH oxidation may be able to interact with the rotenone-resistant pathway of NADH oxidation in the presence

of added NAD (Day and Wiskich, 1974a). The rotenone-resistant NADH dehydrogenase apparently transfers reducing equivalents around the rotenone block and back to the electron transport chain in the area of ubiquinone and cytochrome b (Palmer and Coleman, 1974). However, there is controversy as to the existence of this dehydrogenase. Day and Wiskich (1974a) postulated the presence of an "unidirectional transmembrane transhydrogenase" that was capable of transferring reducing equivalents to the pathway oxidizing exogenous NADH. Later, it was suggested that a lower affinity for rotenone was responsible for the existence of rotenone-resistant oxidation of endogenous NADH (Wiskich and Day, 1979).

In yeast mitochondria two distinct pathways for the oxidation of endogenous NADH have been found. In Saccharomyces species, one pathway is coupled to three sites of phosphorylation and the other pathway is coupled to two sites of phosphorylation (Mackler and Haynes, 1973). Both of these are resistant to rotenone (Ohnishi et al, 1966). The pathway operating is dependent upon the growth phase of the population. The early stationary growth phase corresponds to two sites of phosphorylation whereas the late stationary growth phase corresponds to the operation of all three sites (Mackler and Haynes, 1973; Ohnishi, 1973).

In Candida species of yeast the growth phase again controls the pathway operating (Katz, 1971; Ohnishi, 1973). In this case, though, the pathway coupled to three sites of phosphorylation is rotenone-sensitive (Katz, 1971; Katz, Kilpatrick and Chance, 1971; Ohnishi, 1973).

It now seems fairly certain that two dehydrogenases do exist, one that is rotenone-sensitive and the other that is rotenone-resistant. Study of these two dehydrogenases is integral to the study of rotenone resistance in plant mitochondria.

The Significance of Multiple Pathways of NADH Oxidation. The main metabolic role of NADH is to link the oxidation of various substrates to the formation of ATP, resulting in oxygen consumption. However, the existence of four different dehydrogenases functioning to transfer reducing equivalents suggests additional roles.

The external NADH dehydrogenase on the outer membrane may not function *in vivo* to transfer reducing equivalents to the electron transport chain (Palmer and Coleman, 1974). Both of the external NADH dehydrogenases may regulate the cytosolic ratio of NAD/NADH. This is of importance in the regulation of many metabolic pathways, most notably glycolysis. To maintain glycolytic flux NADH must be reoxidized (Coleman and Palmer, 1972; Marx and Brinkmann, 1978).

Functions of the internal NADH dehydrogenases may be to produce low molecular weight carbon skeletons in the case of the resistant pathway, and to produce ATP in the case of the sensitive pathway (Palmer and Coleman, 1974). Evidence for this comes from studies of yeast mitochondria. The rotenone-resistant pathway operates in cells undergoing rapid growth and thus requiring many carbon skeletons. Some workers have found that low levels of ATP stimulate the rotenone-sensitive pathway (Palmer and Coleman, 1974), thereby increasing ATP production. However, others have found that ATP levels have very little effect on the operation of these pathways (Marx and Brinkmann, 1978). Marx and Brinkmann (1978) have suggested that the rotenone-resistant pathway functions as

an "overflow pipe" for excess NADH. This suggests a role in regulating NAD/NADH in the matrix in the same manner as the external NADH dehydrogenase regulates the cytosolic ratio.

The roles of the dehydrogenases are probably not this simple as the NAD-linked dehydrogenases seem to be kinetically linked with the internal NADH dehydrogenases (Brunton and Palmer, 1973).

Malic Enzyme. A basic difference between plant and animal mitochondria is the ability of plant mitochondria to oxidize malate in the absence of an oxaloacetate removal system (Brunton and Palmer, 1973; Coleman and Palmer, 1972; Ikuma, 1972; Ikuma and Bonner, 1967; Macrae and Moorhouse, 1970; Wiskich and Bonner, 1963). Mitochondria isolated from animal tissue require either glutamate to transaminate oxaloacetate or acetyl CoA to condense oxaloacetate. Plant mitochondria do not require either of these, presumably because of malic enzyme (L-malate:NAD oxidoreductase (decarboxylating) E.C. 1.1.1.39) (Coleman and Palmer, 1971; Macrae and Moorhouse, 1970). However, malic enzyme is found in pigeon liver and bovine adrenal cortex mitochondria (Ikuma and Bonner, 1967; Simpson and Eastabrook, 1969). Malic enzyme catalyses the decarboxylation of malate to produce pyruvate with NAD as the electron acceptor (Coleman and Palmer, 1972; Macrae, 1971; Wiskich and Rayner, 1978). NADP can also function as the electron acceptor (Coleman and Palmer, 1972; Macrae, 1971.

Very little work has been done on the purified enzyme. The Km and Vmax values for malic enzyme purified from Jerusalem artichoke tubers are as follows: Km; 0.57 mM NAD; 0.74 mM NADP; 1.7 mM

L-malate; Vmax in moles NAD(P)H/min/mg protein; 3.96; .94; and 3.98 (Coleman and Palmer, 1972).

Malic enzyme requires Mn++ or Mg++ as a cofactor (Coleman and Palmer, 1972). The enzyme is specific for L-malate, and malonate competitively inhibits its activity (Coleman and Palmer, 1972). The enzyme has very little oxaloacetate decarboxylase activity (Coleman and Palmer, 1972; Macrae, 1971). In some cases oxaloacetate inhibits the enzyme (Brunton and Palmer, 1973). In addition to this NADH competitively inhibits malic enzyme (Coleman and Palmer, 1972; Macrae, 1971). NAD is expected to bind to the enzyme before malate (Coleman and Palmer, 1972).

The pH optimum of malic enzyme is 6.7 to 6.9 (Coleman and Palmer, 1972). Activity decreases rapidly at higher pHs (Arron and Edwards, 1979; 1980; Coleman and Palmer, 1972).

Malic enzyme mediated electron transport has been shown to be inhibited by SHAM, whereas antimycin A, rotenone and cyanide do not cause inhibition (Rustin and Moreau, 1979). Rustin and Moreau (1979) suggest that malic enzyme activity is controlled by the cyanide-resistant pathway and functions as an alternate enzyme for the oxidation of malate.

However, Marx and Brinkmann (1978) found that rotenone-resistant respiration was antimycin A-sensitive. Coleman and Palmer (1972) proposed that malic enzyme functions in the rotenone-resistant pathway. However, later work by Palmer and his colleagues suggested that malic enzyme functions in the rotenone-sensitive pathway (Brunton and Palmer, 1973; Palmer, 1976). Other workers have postulated that malic enzyme is the main dehydrogenase responsible for the oxidation of malate (Douce et al, 1972).

Another area of controversy is the localization of malic enzyme. Initially, many workers felt that it was in the intermembrane space (Coleman and Palmer, 1972; Palmer and Arron, 1976). As malate can be oxidized outside of the inner membrane in the presence of NAD (Coleman and Palmer, 1972; Palmer and Arron, 1976), malic enzyme was expected to be responsible. This too would explain malic enzyme's role in rotenone-resistant respiration, as NADH produced by this enzyme would be oxidized by the external NADH dehydrogenase located on the outside of the inner membrane and linked to two sites of phosphorylation (Coleman and Palmer, 1972; Day and Wiskich, 1974a; 1974b). However, Douce and Bonner (1972) suggested malic enzyme was a soluble matrix enzyme. In retrospect Palmer (1976) also decided that the enzyme was found in the matrix. This presented a problem as NADH produced from malic enzyme would be available to both the rotenone-sensitive and the rotenone-resistant pathways. Thus compartmentation of the matrix was hypothesized. The compartmentation was suggested to be structural (Brunton and Palmer, 1973; Chance and Hollunger, 1961) or kinetic (Palmer and Arron, 1976). Structural compartmentation could result from a structural separation of the NADH dehydrogenases and the close association of malic enzyme and malate dehydrogenase (Brunton and Palmer, 1973). Recent work on the localization of malic enzyme shows that it is a soluble matrix enzyme and it may adhere to the inner membrane (Day, Arron and Laties, 1978).

This section will be dealt with in further detail when the whole system and the interactions are discussed.

Malate Dehydrogenase. Malate dehydrogenase (L-malate: NAD

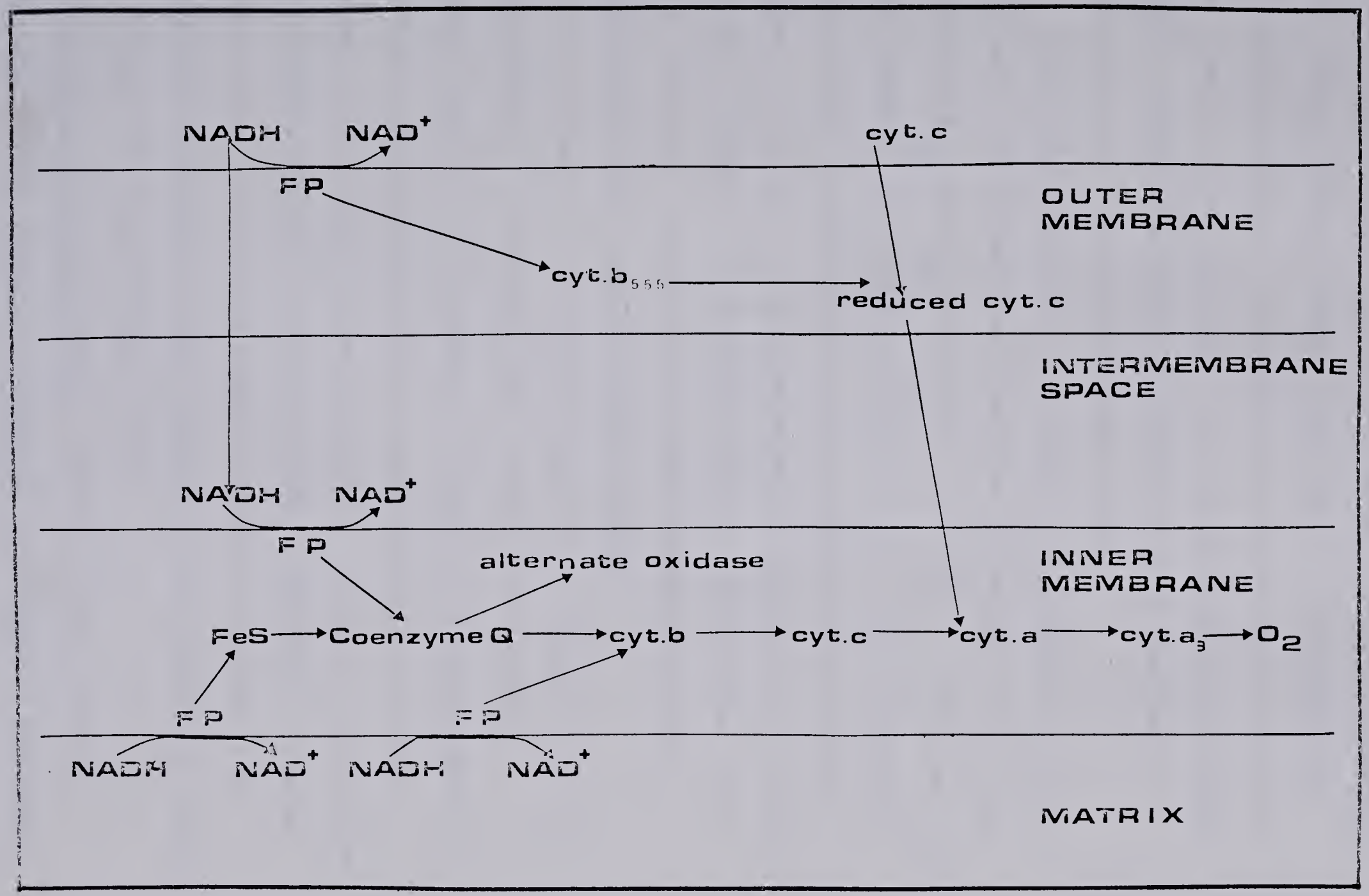
oxidoreductase E.C. 1.1.1.37) is a soluble matrix enzyme that catalyzes the oxidation of malate to oxaloacetate with NAD as the electron acceptor (Coleman and Palmer, 1972; Macrae and Moorhouse, 1970; Palmer and Arron, 1976). This enzyme is found in all mitochondria and was until recently felt to be the main enzyme for the oxidation of malate. It is readily reversible, in fact the thermodynamic equilibrium of the reaction favours the reverse reaction.

At equilibrium the reaction favours oxaloacetate production. The equilibrium constant for malate dehydrogenase is 2.0×10^{-5}. Therefore, high levels of malate are required during oxidation. (Bowman and Ikuma, 1976). Both oxaloacetate and NADH are strong inhibitors of the forward reaction (Hulme, Rhodes and Wooltorton, 1967a; Raval and Wolfe, 1962a; Wiskich and Day, 1979). At pH 6.8, which maximizes the activity of malic enzyme, the formation of malate from oxaloacetate is preferred (Raval and Wolfe, 1962b). The Km of malate oxidation for malate is 5 mM in mung bean mitochondria (Bowman and Ikuma, 1976).

The Electron Transport Chain. The electron transport chain is composed of ubiquinone, the cytochromes and flavoproteins (see Fig. 1). Associated with the chain are three sites of phosphorylation. Ubiquinone is expected to be at the junction of the cyanide-insensitive pathway and the cyanide-sensitive pathway. There may be two pools of ubiquinone, each transferring electrons to one of the above pathways (Storey, 1973).

The cytochrome chain is arranged in decreasing order of redox potential. The number and placement of these has been studied

Fig. 1 A possible organization of the electron transport chain in plant mitochondria.


NADH
NAD+
FP
cyt.c
OUTER MEMBRANE
cyt.b555
reduced cyt.c
INTERMEMBRANE SPACE
NADH
NAD+
FP
alternate oxidase
INNER MEMBRANE
FeS
Coenzyme Q
cyt.b
cyt.c
cyt.a
cyt.a3
O2
FP
FP
NADH
NAD+
NADH
NAD+
MATRIX

extensively and the only area of controversy is the b cytochromes (see Palmer, 1976 for review). The study of the flavoproteins is technically more difficult than the cytochromes and therefore much less is known about them. If a rotenone-resistant pathway exists, the branching point is expected to be in the area of the flavoproteins (Marx and Brinkmann, 1978).

Four flavoproteins have been found to be involved in electron transport. Two of these have high redox potentials and have been designated Fp_{ha} and Fp_{hf} and two have low redox potentials and have been designated Fp_{lf} and Fp_{la} (Storey, 1970). The high potential flavoproteins may be important in the cyanide-resistant pathway (Palmer, 1976), as Fp_{ha} is found between ubiquinone and cytochrome b and Fp_{hf} is found in the cyanide-resistant pathway (Ikuma, 1972). This, of course, is not consistent with ubiquinone being the branch point of the cyanide-resistant pathway.

Fp_{lf} appears to be closely related to the endogenous pyridine nucleotide pool as they both have similar redox potentials (Palmer, 1976). Fp_{la} is reduced by malate via an amytal-sensitive pathway and by reversed electron transport flow from succinate (Palmer, 1976). These two are expected to function in the rotenone-sensitive and rotenone-resistant pathways, respectively. However, recent studies of the redox behavior of these have shown that there is no difference in their response in the presence and absence of rotenone (Marx and Brinkmann, 1978).

The "Rotenone-Resistant Pathway". Work on rotenone-resistant respiration really began in 1972 when it was recognized that there were two NAD-linked enzymes capable of oxidizing malate. Small

quantities of pyruvate were found to be produced from the oxidation of malate in avocado fruit mitochondria (Lance, Hobson, Young and Biale, 1967) and in apple peel mitochondria (Hulme, Rhodes and Wooltorton, 1967b). In cauliflower bud mitochondria pyruvate was the direct product of malate oxidation (Macrae and Moorhouse, 1970). The oxidation of malate to produce pyruvate was linked to the electron transport chain and required NAD. This was also found in mitochondria isolated from Jerusalem artichoke tubers (Coleman and Palmer, 1972). Malic enzyme was responsible for the oxidation of malate to produce pyruvate.

From further work on Jerusalem artichoke and mung bean hypocotyl mitochondria, Coleman and Palmer (1972) found that malate was oxidized via the endogenous NADH pathway of oxidation by malate dehydrogenase and via the exogenous NADH pathway of oxidation by the malic enzyme. It was also found that one of these pathways was coupled to three sites of phosphorylation and was rotenone-sensitive while the other was coupled to two sites of phosphorylation and was rotenone-insensitive.

Malic enzyme was postulated to be in the outer mitochondrial compartment as this was the only way it could reduce exogenous NAD. If NAD was added to rotenone inhibited mitochondria the inhibition was alleviated. From this they determined that malic enzyme was associated with the rotenone-resistant pathway.

The pathway suggested by Coleman and Palmer would allow for the production of NADH and pyruvate in the intermembrane space. This is made possible by the translocation of malate to the outer compartment.

The level of NADH would be controlled by the action of the exogenous NADH dehydrogenase. As malic enzyme is inhibited by NADH, the action of these two enzymes could aid in the control of the cytosolic NAD/NADH.

Douce et al (1972) later suggested that malic enzyme was the main enzyme for the oxidation of malate. Further to this, they suggested that malic enzyme utilized a common pool of NAD and therefore was within the inner mitochondrial membrane.

In response to this Brunton and Palmer (1973) did further work on the localization and inhibition of malate oxidation. Ferricyanide reduction by the piericidin-A-resistant pathway was found to be antimycin A-sensitive. This further suggested that malic enzyme was found in the matrix .

As oxaloacetate will readily inhibit pyruvate or citrate oxidation but will not inhibit malate oxidation the idea of compartmentalization was developed (Palmer and Arron, 1976; Brunton and Palmer, 1973; Palmer, Cowley and Al-Sane, 1978). Evidence to support this theory included the reduction of endogenous NAD in two phases (Brunton and Palmer, 1973). This is consistent with the idea of two pools of NAD.

Malic enzyme was expected to be associated with the rotenone-sensitive pathway and malate dehydrogenase was expected to be associated with the rotenone-resistant pathway.

Day and Wiskich (1974a) looked further at the pathways of malate oxidation in cauliflower bud mitochondria. Malate oxidation was altered by the addition of NAD. This caused oxidation similar to NADH oxidation and almost completely relieved rotenone inhibition.

This was explained by the presence of a hypothetical "unidirectional transmembrane transhydrogenase" (Day and Wiskich, 1974a; 1974b). This would reduce NAD which would then be reoxidized via the external NADH dehydrogenase on the outside of the inner membrane (Day and Wiskich, 1974b). An alternate explanation of this was developed by Neuburger and Douce (1978). They noted that NAD had little effect when there was high endogenous NAD. Also, oxaloacetate inhibition of malate oxidation did not increase when there was exogenous NAD. They therefore explained the effect of NAD as stimulating malic enzyme activity, thus increasing the rate of oxaloacetate removal. This is supported by the work of Spalding, Arron and Edwards (1980) in which oxaloacetate or NAD stimulated malic enzyme activity. The buildup of NADH during rotenone inhibition also supports this idea (Wiskich and Day, 1979).

The major discrepancy between the results of Day and Wiskich and Palmer and his co-workers are: Day and Wiskich found that n-butyl malonate (an inhibitor of the phosphate-malate exchange carrier in animal mitochondria (Robinson and Chappell, 1967)) inhibited malate oxidation in the presence of NAD and rotenone; and Day and Wiskich reported a requirement for glutamate to maintain the rate of malate oxidation. Glutamate may affect the respiration rate as it alters the balance of substrates (Arron and Edwards, 1979).

In 1976, Palmer and Arron (1976) suggested there were two internal pathways of malate oxidation, one which was rotenone-sensitive and the other which is rotenone-resistant. The sensitive

pathway was expected to be directly available to malate dehydrogenase and the resistant pathway was expected to be directly available to malic enzyme (Palmer and Arron, 1976).

More recent work by Day and Wiskich (1979) makes no mention of the "unidirectional transmembrane transhydrogenase" and in fact suggests that a rotenone-resistant pathway may not even exist at all. However, they expect the presence of the pathway to be the most likely explanation.

An interesting observation is made in the paper. The "recovered rate of rotenone inhibition" (the rate of oxygen consumption in State 3 following the transient inhibition) was equivalent to the inhibited rate for other NAD-linked substrates. This suggests the existence of more than one site of rotenone inhibition. The NADH dehydrogenase probably accounts for the permanent inhibition or recovered rate and one of the malate dehydrogenases accounts for the transient inhibition. Malate dehydrogenase was expected to be the inhibited oxidoreductase (Wiskich and Day, 1979).

The basis for the assumption is that both NADH and oxaloacetate accumulated when rotenone is added (Wiskich and Day, 1979). This would cause malate dehydrogenase to reverse thus oxidizing NADH and producing malate. However, oxaloacetate has been shown to affect all NAD-linked substrates equally (Douce and Bonner, 1972). As rotenone appears to affect malate oxidation to a greater degree than it affects the oxidation of other NAD-linked substrates (Wiskich and Day, 1979), oxaloacetate could not be responsible for this effect. Further to this

oxaloacetate has not been shown to accumulate in rotenone inhibited mitochondria (Brunton and Palmer, 1973).

The accumulation of NADH has been shown to occur by a number of workers (Coleman and Palmer, 1972; Day and Wiskich, 1979). This could inhibit malate dehydrogenase activity thus leading to more oxidation of malate via malic enzyme. However, malic enzyme is also inhibited by NADH (Coleman and Palmer, 1972), therefore, permanent inhibition of these enzymes would occur.

Rustin and Moreau (1979) have suggested that the rotenone-resistant pathway is mediated by the cyanide-resistant pathway and involves malic enzyme. As rotenone-resistant respiration is coupled to two sites of phosphorylation and cyanide-resistant respiration is not associated with ATP production, this seems unlikely. Also rotenone-resistance has been shown to be antimycin A-sensitive (Marx and Brinkmann, 1978).

NAD has been shown to alleviate rotenone inhibition (Brunton and Palmer, 1973; Coleman and Palmer, 1972; Wiskich and Day, 1979). This along with the fact that NADH accumulates in rotenone inhibited mitochondria (LaNoue, Bryla and Williamson, 1972; Ragan and Garland, 1971; Oberg, 1971; Wiskich and Day, 1979) suggests that rotenone inhibition is caused by an imbalance of NAD/NADH, rather than an imbalance occurring due to rotenone inhibition. NAD is transported through the inner mitochondrial membrane (Neuburger and Douce, 1978). NAD has the greatest effect in tissue which has low endogenous levels of NAD (Neuburger and Douce, 1978). Therefore the exogenously applied NAD may hasten the rate at which the NAD/NADH ratio is re-established. As NAD preferentially

increases the rate of oxaloacetate production, the equilibrium effect alone could account for the inhibition of malate dehydrogenase (Bowman and Ikuma, 1976b). The bypass of the first site of phosphorylation could be accounted for by the movement of NADH to the external NADH dehydrogenase on the outside of the inner membrane.

MATERIALS AND METHODS

Plant Material. Pea seeds (Pisum sativum L. var Homesteader) were soaked in tap water for six hours. Damaged and nonimbibed seeds were discarded and the remainder were planted in horticultural grade vermiculite. The seeds were germinated at 27 C in the dark for various periods.

The method of isolation was basically that of Solomos et al (1972).

Isolation of Mitochondria. Approximately 250 ml of washed cotyledons were ground with a mortar and pestle for 7 min in 250 ml of ice cold extraction medium: 0.5 M mannitol; 5 mM EDTA; 0.5% fatty acid poor BSA; 0.05% cysteine and 0.05% Tes. The pH was adjusted to 7.1 at 25 C with KOH. The brei was filtered through one layer of miracloth and the filtrate was then centrifuged at 700 g for 7 min in a Beckman JA-20 rotor. The pellet was discarded and the supernatant layer was centrifuged at 21,000 g for 5 min in a Beckman JA-20 rotor. The pellet was resuspended in 50 ml of ice cold, wash medium (0.3 M mannitol, 25 mM Tes and 0.3% fatty acid poor BSA, the pH adjusted to 7.1 at 25 C with KOH). Centrifugation was at 21,000 g for 5 min in a Beckman JA-20 rotor. The pellet was resuspended in 3 ml of suspend medium (0.3 M mannitol, 4 mM $MgCl_2$ and 25 mM Tes, adjusted to pH 7.1 at 25 C with KOH) and loaded onto a sucrose density gradient (26 ml 0.6 M sucrose and 10 ml 1.6 M sucrose, both with 50 mM Tes and 0.1% BSA adjusted to pH 7.1 at 25 C with KOH) or used as such for mitochondria prepared

by differential centrifugation. This was centrifuged for one hour at 30,000 g in a Beckman SW27 rotor. The fraction at the interface was removed and diluted slowly with a buffered solution (25 mM Tes and 0.1% BSA, adjusted to pH 7.1 at 25 C with KOH). The fraction was then centrifuged for 5 min at 21,000 g in a Beckman JA-20 rotor and the pellet was resuspended in the suspend medium used above. All steps of the isolation were carried out at 0 to 4 C.

Respiratory Measurements. Respiratory measurements were made with a Yellow Springs Instrument Company Model 53 oxygen monitor connected to a Beckman 100 mV potentiometric recorder. The malate reaction medium consisted of 0.3 M mannitol, 50 mM Tes, 0.4 mM $MgCl_2$, 5 mM primary potassium phosphate, 0.75 mg/ml BSA and 8 mM malate (Solomos et al, 1972). The pH was adjusted to 7.1 at 25 C with KOH.

The α-ketoglutarate reaction medium and the procedures were those of Malhotra and Spencer (1970). When inhibitors were used they were added to State 4 mitochondria after 2 cycles of ADP addition. Approximately 150 nmol of ADP was then added. The inhibitor resistant rate was taken to be the rate following the ADP addition.

When more than one inhibitor was to be used approximately 300 nmol ADP was added after addition of the first inhibitor. After a steady oxygen uptake rate had been established the second inhibitor was added. The recovered rate was determined by the addition of 150 nmol ADP following the return to State 4 after the inhibited State 3.

Succinate-Cytochrome c Reductase. Succinate-cytochrome c reductase activity was measured with a Cary 15 recording spectrophotometer at 550 nm according to the method of Douce et al (1972): 5 mM phosphate buffer (pH 7.2), 0.05 mM cytochrome c, 1 mM KCN and 0.1 - 1 mg of mitochondrial protein. The reaction was initiated with 10 mM succinate.

NADPH-Cytochrome c Reductase. NADPH-cytochrome c reductase activity was measured with a Cary 15 recording spectrophotometer at 550 nm according to the method of Douce et al (1972): 5 mM phosphate buffer (pH 7.2), 0.05 mM cytochrome c, 1 mM KCN and 0.1 - 1 mg of mitochondrial protein. The reaction was initiated with 0.3 mM NADPH. When antimycin A was used it was added before the mitochondria to give a final concentration of 0.4 ug/ml.

Purification and Characterization of the End Product Formed from Rotenone. Rotenone was added to active or heat denatured mitochondria that had been suspended in malate assay medium or distilled water. Mixing was continued until the transient inhibition had ceased. Aliquots were taken, boiled and then centrifuged for 3 min at full speed on an International Model HN centrifuge. The rotenoids were then extracted from the pellet according to the methods of Payfer (1954). Paper chromatography of the products was according to the methods of Delfel (1965). The purity of the product was monitored by scanning from 860 nm to 260 nm on a Cary 15 recording spectrophotometer.

Isolation and Purification of Enzymes. Malate dehydrogenase was purified and assayed according to the methods of Ochoa (1955). For the preparation of malic enzyme, mitochondria from approximately

500 ml of cotyledons were purified as for mitochondria isolated by differential centrifugation, except that the mitochondria were finally suspended in the sonication medium of Coleman and Palmer (1972).

The method of purification and assay were basically that of Coleman and Palmer (1972). Changes were as follows; centrifugation was for one hour at 5900 g; and the enzyme was not concentrated. Either NAD or NADP was used as the cofactor of the reaction. Varying concentrations of rotenone and elliptone were added to both enzyme preparations.

Spectrophotometry of Respiratory Pigments. Mitochondria were prepared as above and the protein content was adjusted such that there was 0.5 mg protein/ml of assay medium. The assay medium was as above except that the malate concentration was increased to 80 mM. The reaction was initiated by the addition of 3 mM ADP. Scans of the pigments were done according to the methods of Chance (1957), with a Cary 15 recording spectrophotommter. The scan rate was approximately equal to one spectral band width per period, assuming that the spectral band width was approximately one-tenth the natural band width of the smallest absorption band. Cyanide, as KCN (final concentration 5 mM) and rotenone (final concentration 13.3 μM) were added where indicated.

Assay of Reduction Products. The products of malate oxidation were measured over a period of 20 min using the same assay medium and protein concentration as in the spectrophotometry of respiratory pigments. Oxygen consumption was measured simultaneously in a separate sample. Rotenone (13.3 μM) was added. Aliquots of 1 ml were taken

every 5 min and the reaction in the aliquot was stopped by the addition of 100 µl of 50% perchloric acid. The resulting mixture was centrifuged for 3 min at full speed in an International Model HN centrifuge. Then the supernatant layer was adjusted to pH 5 to 6 with 5 M K_2CO_3 (Coleman and Palmer, 1972). This mixture was centrifuged as before and the supernatant layer was assayed for the presence of oxaloacetate (Williamson and Corkey, 1969) and pyruvate (Von Vorff, 1969).

Protein. Protein was determined according to the method of Sedmak and Grossberg (1977).

Statistics. In all data sets showing the difference between treatments the students t distribution for paired sample hypothesis was used with the α-level at 0.05 (Zar, 1974). Linear regressions were used to determine the best line and the fit of that line for all enzyme data (Zar, 1974).

Chemicals. All chemicals were of reagent grade and with the exception of the following were from Fisher Scientific Co. Cytochrome c (Type III), L-cysteine, glycylglycine, rotenone, Tes, L-malate, pyruvic acid, antimycin A, NAD, NADP, NADH, ADP, malate dehydrogenase and lactic acid dehydrogenase were from Sigma Chemical Co. and BSA, dithiothreitol and oxaloacetate were from Calbiochem. The dialysis tubing used was Spectropore, mol wt cutoff 6,000 to 8,000.

RESULTS

Preparative Methods. Mitochondria prepared by a sucrose density gradient had higher rates of oxygen consumption than those prepared by differential centrifugation (Table I). ADP:O ratios were markedly higher while the respiratory control ratios were slightly lower. The percentage of rotenone-resistant respiration was much lower and less variable in the gradient isolated mitochondria. Both NADPH-cytochrome c reductase and succinate-cytochrome c reductase activities were low. Both preparations produced mitochondria that were antimycin A-sensitive. Mitochondria isolated by simple differential centrifugation were found to lose respiratory control over a relatively short period of time compared to those isolated on the density gradient.

The Effects of Rotenone on α-Ketoglutarate and Malate Oxidation. Mitochondria utilizing α-ketoglutarate as the respiratory substrate were very rotenone-resistant (Fig. 2) (Table II). Rotenone (20 μM) caused maximum inhibition in all ages of mitochondria. Four day old mitochondria maintained respiratory control at this concentration. The ADP:O ratio was lowered by one unit in the presence of rotenone (from 2.5 to 1.59). The ADP:O ratios for other ages could not be determined by the polarographic method as there was no return to State 4 following rotenone inhibition.

Rotenone concentrations as low as 0.66 μM caused inhibition of State 3 respiration in mitochondria when malate was the substrate (Fig. 3: A and B). Maximum inhibition was caused by 6.7 μM. State 4 respiration was not affected by concentrations in the range used.

Table I. A Comparison of the Respiratory Activities, Intactness and Response to Rotenone of Pea Cotyledon Mitochondria Prepared by Differential Centrifugation and by Sucrose Density Gradient Centrifugation.

Mitochondria were prepared from 6 day old pea cotyledons as in the Materials and Methods section. Values shown are the means of 3 separate preparations. The mean deviation was ± 10%.

	Differential	Density Gradient
Oxygen uptake, State 3 (n atoms/min/mg protein)	98.6	150
ADP:O ratio	1.3	1.9
Respiratory control ratio	3.8	3.5
Succinate-cytochrome c reductase (nmoles cytochrome c reduced/min/ mg protein)	4.6	3.6
NADPH-cytochrome c reductase (nmoles cytochrome c reduced/min/ mg protein)[a]	20.2	22.4
Antimycin A-resistant NADPH cytochrome c reductase (nmoles cytochrome c reduced/min/mg/protein)	5.0	2.9
Rotenone resistant respiration	58 %	33 %
Rotenone concentration causing maximum inhibition (μM)	3.3	3.3

[a] The millimolar absorbance coefficient at 550 nm was 21.0 $mM^{-1}cm^{-1}$

Assay medium: 0.3 M mannitol, 50 mM Tes, 0.4 mM $MgCl_2$, 5 mM potassium phosphate (monobasic), 0.75 mg/ml BSA and 8 mM malate, pH adjusted to 7.1 at 25 C with KOH, final volume 3.2 ml.

Fig. 2 The effect of rotenone on the respiratory activity of pea cotyledon mitochondria oxidizing α-ketoglutarate. Procedure as in Materials and Methods. Each point represents the mean of three different preparations. The mean deviation was ± 9%.

--⊟--- 3 day old

—▶— 4 day old

--⊖-- 5 day old

—●— 6 day old

—■— 7 day old

Assay medium : 0.3 M mannitol, 50 mM Tes, 0.4 mM $MgCl_2$, 5 mM potassium phosphate (monobasic), 0.75 mg/ml BSA, 5 mM malonate, 0.07 mM thiamine pyrophosphate and 8 mM α-ketoglutarate, pH adjusted to 7.1 at 25 C with KOH, final volume 3.2 ml.

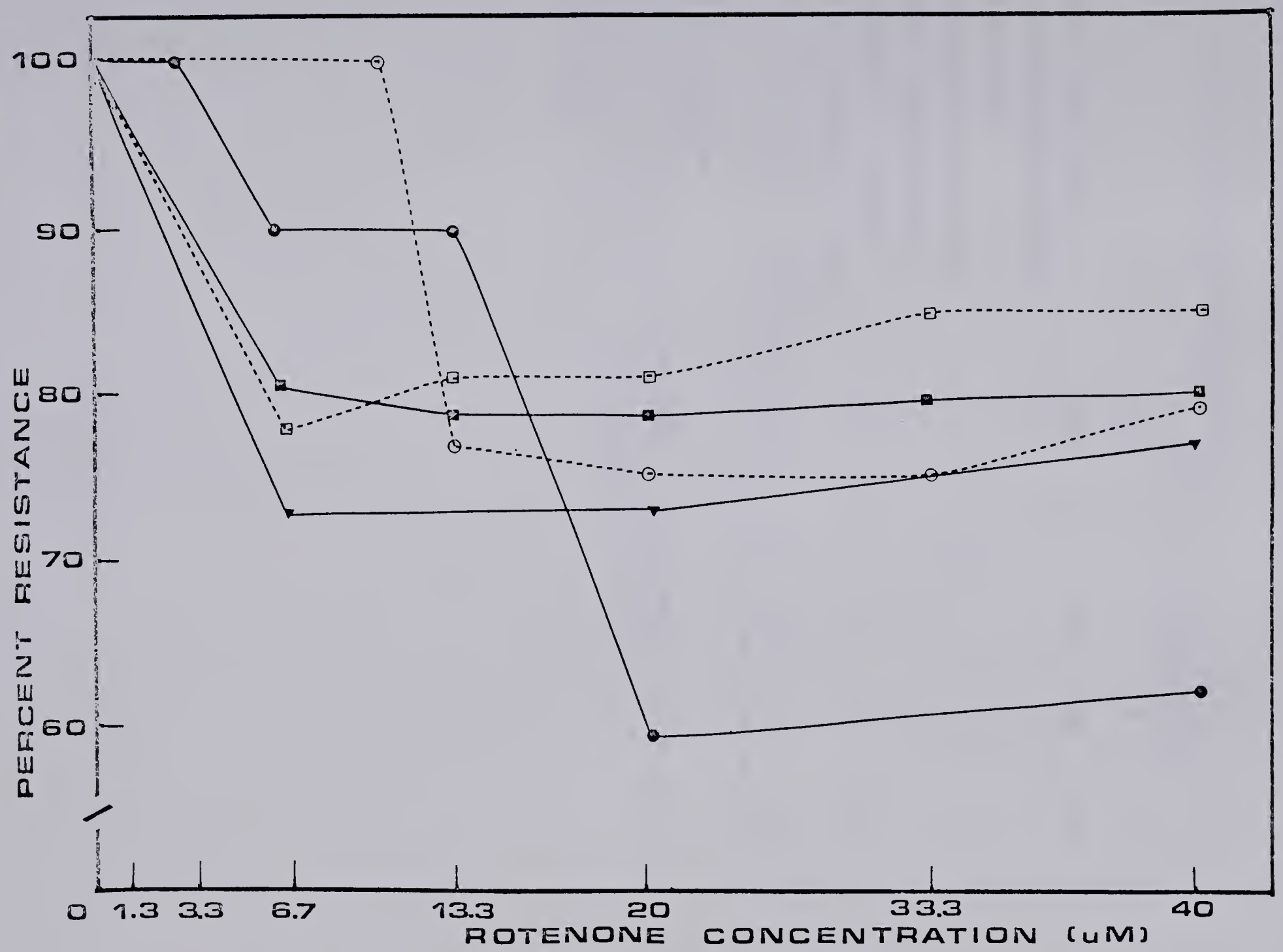

100
90
80
70
60
PERCENT RESISTANCE
0
1.3
3.3
6.7
13.3
20
33.3
40
ROTENONE CONCENTRATION (uM)

Table II. The Effect of Rotenone on Respiratory Activity of Pea Cotyledon Mitochondria Oxidizing α-Ketoglutarate.

	Age of Cotyledons (Days)				
	3	4	5	6	7
Oxygen uptake State 3 (n atoms/min/mg protein)	256	256	200	236	238
ADP:O ratio	2.6	2.5	1.7	2.2	3.5
Respiratory control ratio	2.7	2.8	3.3	2.4	3.1
Oxygen uptake in the presence of rotenone[a] (n atoms/min/mg protein)	222	197	150	141	188
Rotenone-resistant respiration	80%	76%	75%	60%	79%

[a] 20 μM rotenone

Procedures in Materials and Methods. Values shown are the mean of 3 different preparations. The mean deviation was ± 9.%.

Assay medium: As in Fig. 2 .

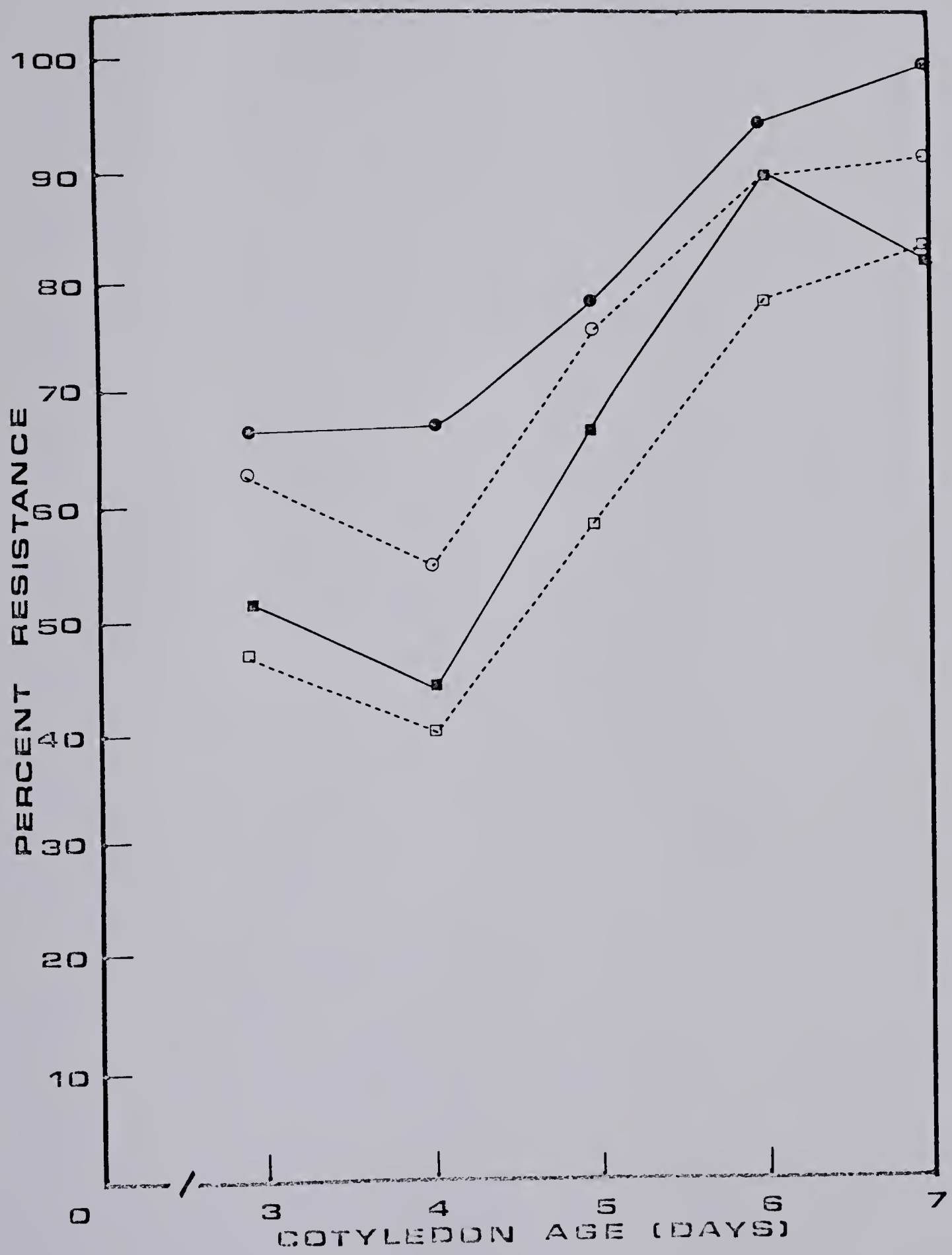
Fig. 3: A and B. The effect of cotyledon age on the resistance of mitochondrial respiration to rotenone. Procedures for the isolation of the mitochondria as in Materials and Methods. Each value represents the mean of three separate experiments. The mean deviation was ± 10%. 100% represents an oxygen uptake rate of 140 to 200 natoms/min/mg protein.

A. —●— 0.67 µM rotenone --⊖-- 1.0 µM rotenone

—■—1.3 µM rotenone --⊟-- 2.0 µM rotenone

ADP:O ratios for this data set were all approximately 1.9 - 2.1.

Assay medium: 0.3 M mannitol, 0.4 mM $MgCl_2$, 0.75 mg/ml BSA, 5 mM potassium phosphate (monobasic) and 8 mM malate, adjusted to pH 7.1 at 25 C with KOH, final volume 3.2 ml.


PERCENT RESISTANCE
100
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
COTYLEDON AGE (DAYS)

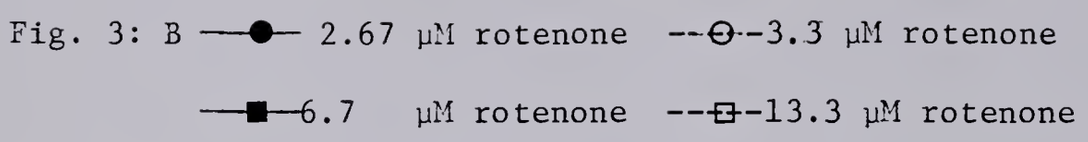
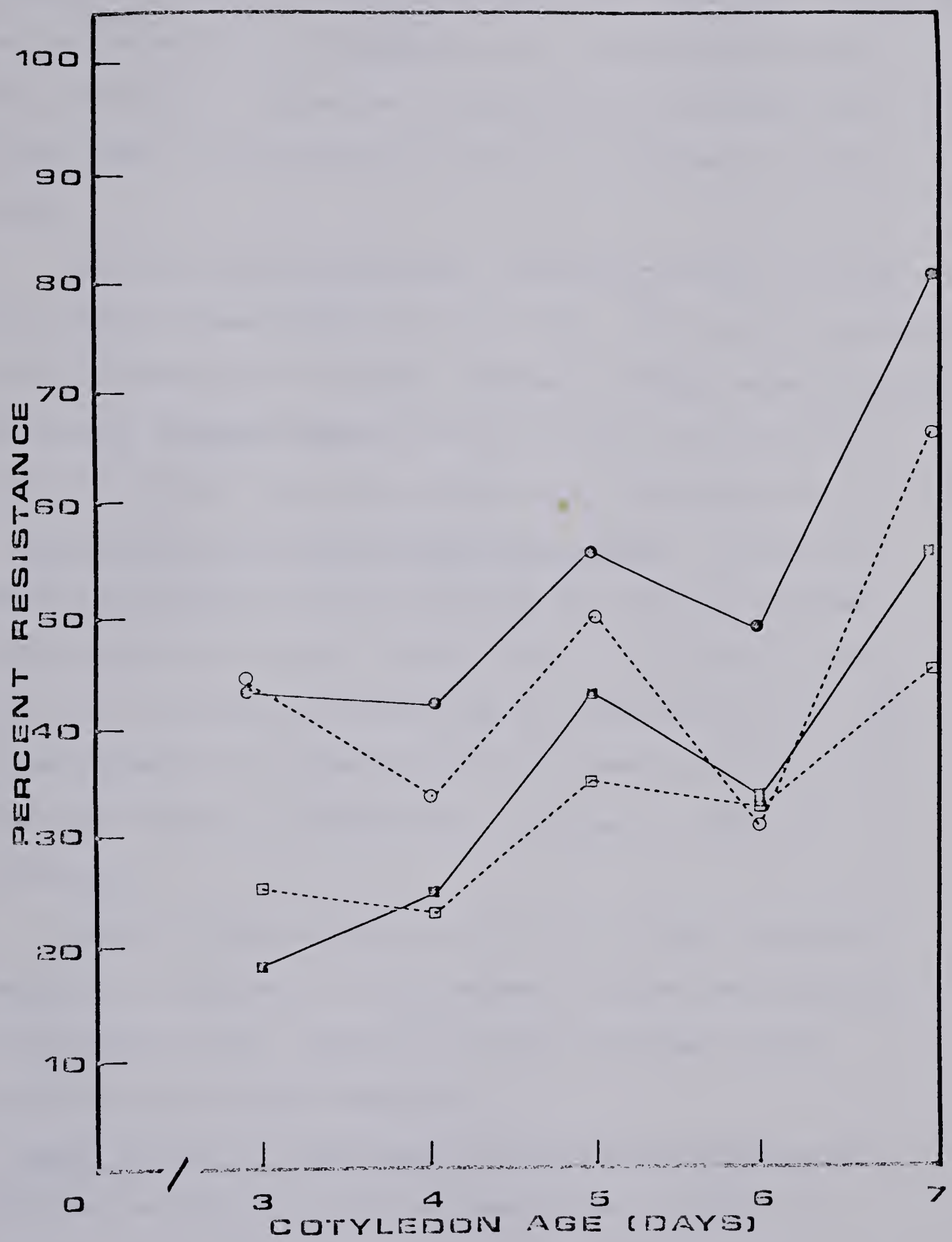


Fig. 3: B
2.67 µM rotenone
3.3 µM rotenone
6.7 µM rotenone
13.3 µM rotenone


PERCENT RESISTANCE
100
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
COTYLEDON AGE (DAYS)

Rotenone-resistance was correlated to the age of the mitochondria and the respiratory control ratio (Fig. 4). Over a wide concentration of rotenone two separate trends in resistance were noted (Fig. 3:A and B). At low concentrations of rotenone six day old mitochondria had high resistance in comparison to mitochondria of other ages, but at the higher concentrations of rotenone the resistance was much lower.

Both malate and α-ketoglutarate oxidation recovered following the initial rotenone inhibition (Table III). The recovery decreased as the concentration of rotenone increased. Although malate oxidation was greatly inhibited initially (Fig. 3:A and B), the recovered rate was much the same as the α-ketoglutarate recovered rate.

Localization of the "Rotenone-Resistant Pathway". Addition of SHAM to mitochondria isolated from seven day old tissue resulted in 44% inhibition of oxygen uptake (Table IV). If SHAM was added after 13.3 μM rotenone had been added the inhibition was 69%. This is approximately the amount of inhibition caused by rotenone alone. Reversing the order of addition did not change the degree of inhibition.

Cyanide (as KCN) concentrations of 5 μM or higher caused 63% inhibition. Addition of 13.3 μM rotenone increased the inhibition to approximately 91%. Addition of cyanide following rotenone addition resulted in the same effect.

Enzyme Inhibition. Malic enzyme (L-Malate NAD:oxidoreductase (decarboxylating)E.C.1.1.1.39) was competitively inhibited by rotenone concentrations of 1.66 μM to 6.6 μM with no further effects at concentrations of inhibitor up to 13.3 μM (Fig. 5).

Fig. 4 Changes in the respiratory control ratios and in maximum inhibition by rotenone of respiration of mitochondria from pea cotyledons of different ages. Mitochondria from pea cotyledons were isolated and assayed as in Materials and Methods. Each value represents the mean of three separate experiments. The mean deviation was $\pm$ 10 %. 50% inhibition represents an oxygen uptake rate of 70 to 100 natoms/min/mg protein.

—●— Percent inhibition = $\frac{\text{State 3 } O_2 \text{ uptake in the presence of 13.3 } \mu M \text{ rotenone}}{\text{State 3 } O_2 \text{ uptake prior to the addition of rotenone}}$

—⊖— Respiratory control ratio

Assay medium: As in Fig. 3 .

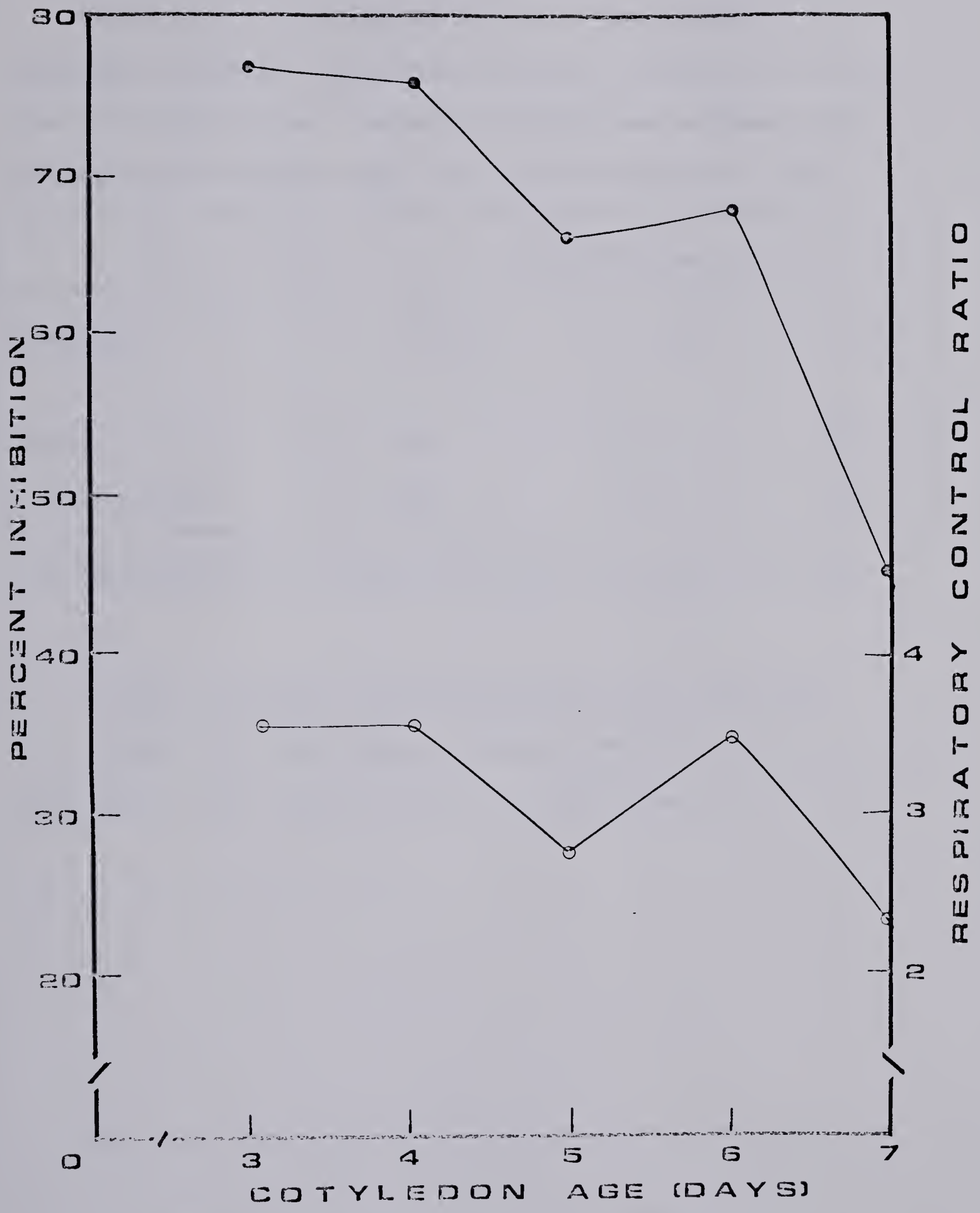

80
70
60
50
40
30
20
0
3
4
5
6
7
PERCENT INHIBITION
RESPIRATORY CONTROL RATIO
4
3
2
COTYLEDON AGE (DAYS)

Table III. Recovered Rate[a] of Respiration in Pea Cotyledon Mitochondria Oxidizing Malate or α-Ketoglutarate in the Presence of Rotenone.

Mitochondria from 5 day old cotyledons were isolated as in Materials and Methods. Each value represents the mean of at least 3 separate preparations. Comparable results were obtained from mitochondria of different ages. The mean deviation was ± 10%.

	Rotenone Concentration		
Substrate	3.3 μM	6.6 μM	13.3 μM
Malate	100%	87%	72%
α-Ketoglutarate	100%	93%	75%

[a]The recovered rate of rotenone inhibition was taken to be equal to:

$$\frac{\text{State 3 } O_2 \text{ uptake following rotenone inhibition X 100}}{\text{State 3 } O_2 \text{ uptake prior to rotenone inhibition}}$$

Assay Media: As in Table II and Fig. 2 and 3:A and B.

Table IV. Rotenone Resistance, in the Presence of Cyanide or SHAM, of Pea Cotyledon Mitochondria Oxidizing Malate.

Mitochondria were isolated from 7 day old pea cotyledon, as described in Materials and Methods. Reaction mixture as in Fig. 3:A and B. Where present, 5 mM KCN was added and 1.5 μM SHAM was added. The inhibited rates were measured as soon as a steady rate of oxygen uptake had been established. The second inhibitor was added after a steady rate of oxygen consumption was established. Each value represents the mean of 3 separate experiments.*

Order of addition	Inhibitor	Percent Inhibition	Inhibitor	Percent Inhibition
1	SHAM	44	Cyanide	63
2	Rotenone	83.8	Rotenone	91
Inhibition caused by 2nd inhibitor[a]		71		76
1	Rotenone	76	Rotenone	74
2	SHAM	69	Cyanide	90.4
Inhibition caused by 2nd inhibitor		0		63

[a] $\text{Inhibition caused by 2nd inhibitor} = \frac{100 - \text{percent inhibition to 2nd inhibitor}}{100 - \text{percent inhibition to 1st inhibitor}} \times 100$

* The mean deviation was ± 9%.

Fig. 5 Lineweaver-Burk plot for the inhibition of malic enzyme by rotenone and elliptone.

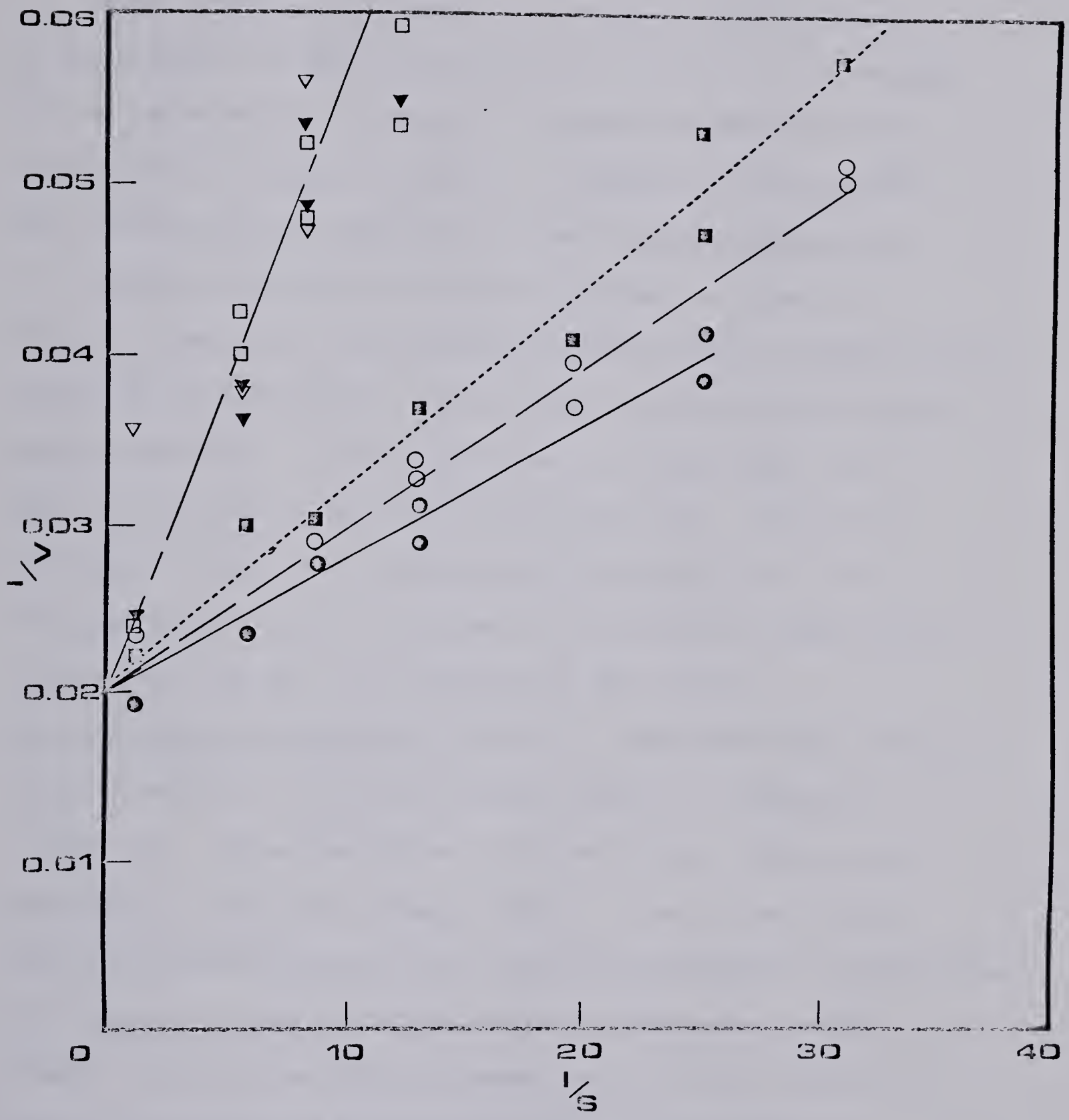
Malic enzyme was prepared and assayed as in Materials and Methods. 1/S represents mmoles NAD/100 sec/mg protein^{-1} and 1/V represents µM NADH^{-1}.

———, ● uninhibited enzyme; — —, ○ 1.67 µM rotenone; -----, ■ 3.3 µM rotenone; — – —, □ 6.7 µM rotenone — – —, ▽ 13.3 µM rotenone; — – —, ▼ 13.3 µM elliptone. *

Significance of the lines: uninhibited enzyme: 0.79, 1.67 µM rotenone: 0.91, 3.3 µM rotenone: 0.87, 6.7 µM rotenone: 0.74, 13.3 µM rotenone: 0.73, 13.3 µM elliptone elliptone: 0.92 (Zar, 1974).

Assay medium: 20 mM Tes, 5 mM $MgCl_2$, 0.3 mM dithiothreitol and 8 mM malate, adjusted to pH 6.8 with KOH at 25 C. The reaction was started by the addition of NAD. The final volume was 3 ml.

* Each point represents the mean of three separate preparations. The mean deviation was $\pm$ 0.009 µM NADH^{-1}.


0.06
0.05
0.04
0.03
0.02
0.01
1/v
0
10
20
30
40
1/s

This is in agreement with the data from isolated mitochondria. Malic enzyme was not inhibited by rotenone when NADP was used as the substrate (Table V).

Malate dehydrogenase activity was measured using an excess of oxaloacetate and NADH. Concentrations as high as 6.7 µM rotenone did not cause inhibition (Table V). No change in inhibition was noted from 13.3 µM to 50 µM rotenone. Therefore it was concluded that rotenone did not inhibit the action of malate dehydrogenase.

The Km and Vmax values for the two enzymes are shown in Table V. From this it can be seen that NADP and NAD are both effective substrates for malic enzyme. The students t distribution for paired samples showed that rotenone inhibition was insignificant when NADP was the cofactor ($t(2)\ 11 \leq 2.29$) (Zar, 1974). The $K\underline{I}$ for malic enzyme inhibited by rotenone was calculated to be 6.6 uM rotenone (Fersht, 1977). Measurement of inhibition by concentrations greater than this were not possible with this method.

The Deactivation of Rotenone. Rotenone is transformed into elliptone in the presence of both active and heat denatured mitochondria (Fig. 6). Scans from 860 nm to 260 nm show the difference in absorbance of these two compounds (Fig. 7). Also it can be seen that the elliptone preparation was free from mitochondrial contamination. Both compounds absorb to the same degree at 340 nm when in 95% ethanol. There was no shift in absorption when either buffers or malate assay medium was used as the solvent. Measurements of NAD reduction would be erroneous otherwise.

Elliptone inhibits mitochondrial respiration. The degree of resistance is higher than when rotenone is the inhibitor (Fig. 8).

Table V. Km and Vmax Values for Malic Enzyme and Malate Dehydrogenase Purified from Pea Cotyledons.

Purification and assay of the enzymes as in Materials and Methods. Both enzymes were prepared from pea cotyledons between the ages of 3 and 5 days.

Substrate	Malic Enzyme		Malate Deydrogenase	
	Km[a]	Vmax[b]	Km[a]	Vmax[b]
NAD	0.04	50	1	100
NAD + 1.66 µM rotenone	0.05	50	1	100
NAD + 3.33 µM rotenone	0.06	50		
NAD + 6.6 µM rotenone	0.185	50	1	100
NAD + 13.3 µM rotenone	0.185	50	0.33	33.3
NADP	6.0	50		
NADP + 13.3 µM rotenone	21	100		

[a] Km as mmolar substrate

[b] Vmax as µmoles product/100 sec/ mg protein

Fig. 6 Paper chromatography of rotenone and elliptone.

Method of purification and solvent system as in Materials and Methods.

A. Rotenone standard; B. Active mitochondria and rotenone in malate assay medium; C. Active mitochondria and rotenone in distilled water; D. Heat denatured mitochondria and rotenone in malate assay medium; E. Malate assay medium and rotenone; F. Heat denatured mitochondria and rotenone in distilled water.

Malate assay medium: As in Fig. 3 .

A. B. C. D.

Fig. 6 Paper chromatography of rotenone and elliptone.

E. Malate assay medium and rotenone.

F. Heat denatured mitochondria and rotenone in distilled water.

E.

F.

Fig. 7 Spectrophotometer scans of rotenone and elliptone in 95% ethanol.

The elliptone was purified according to the procedure outlined in Materials and Methods.

———— 13.3 μM rotenone ------- elliptone

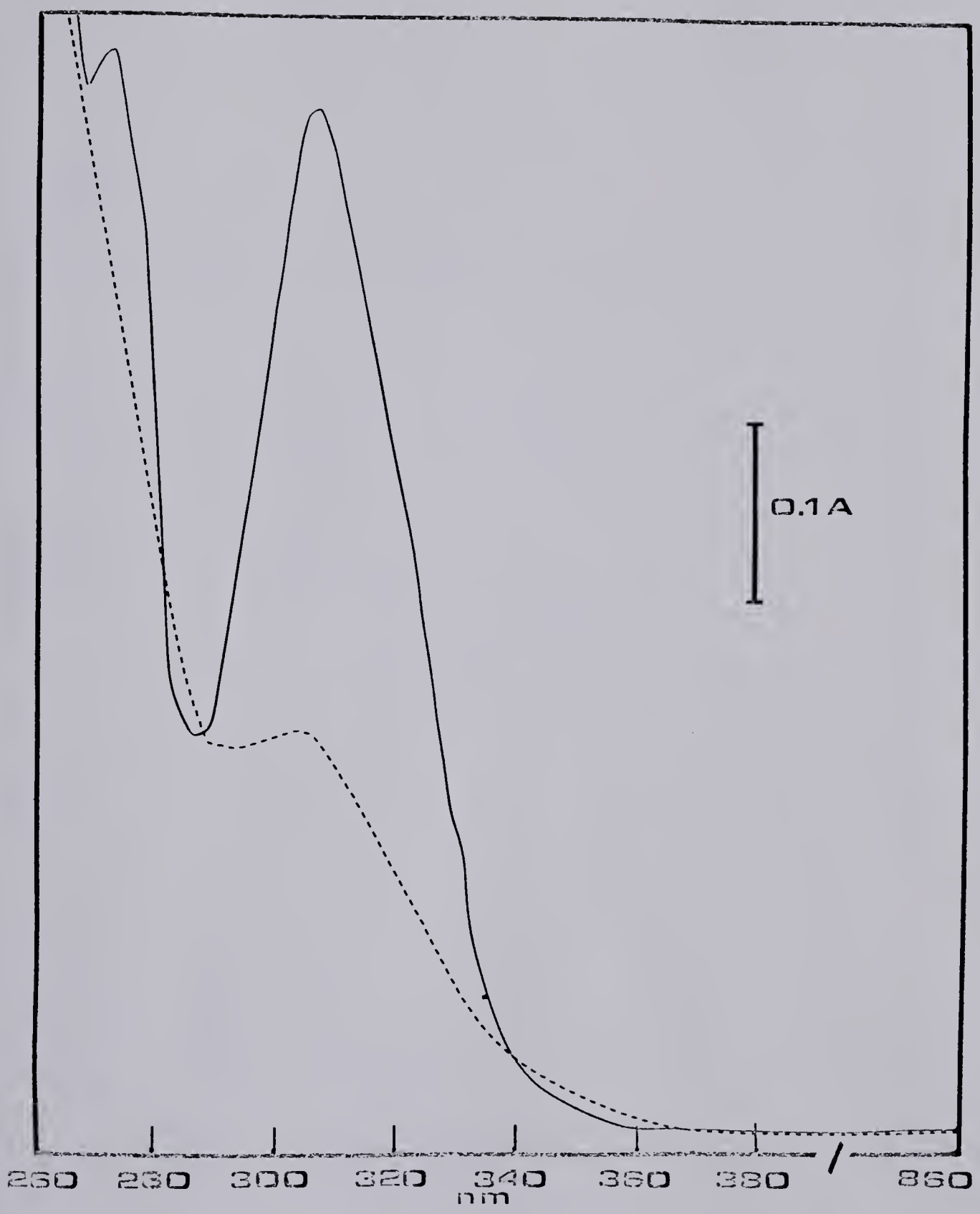
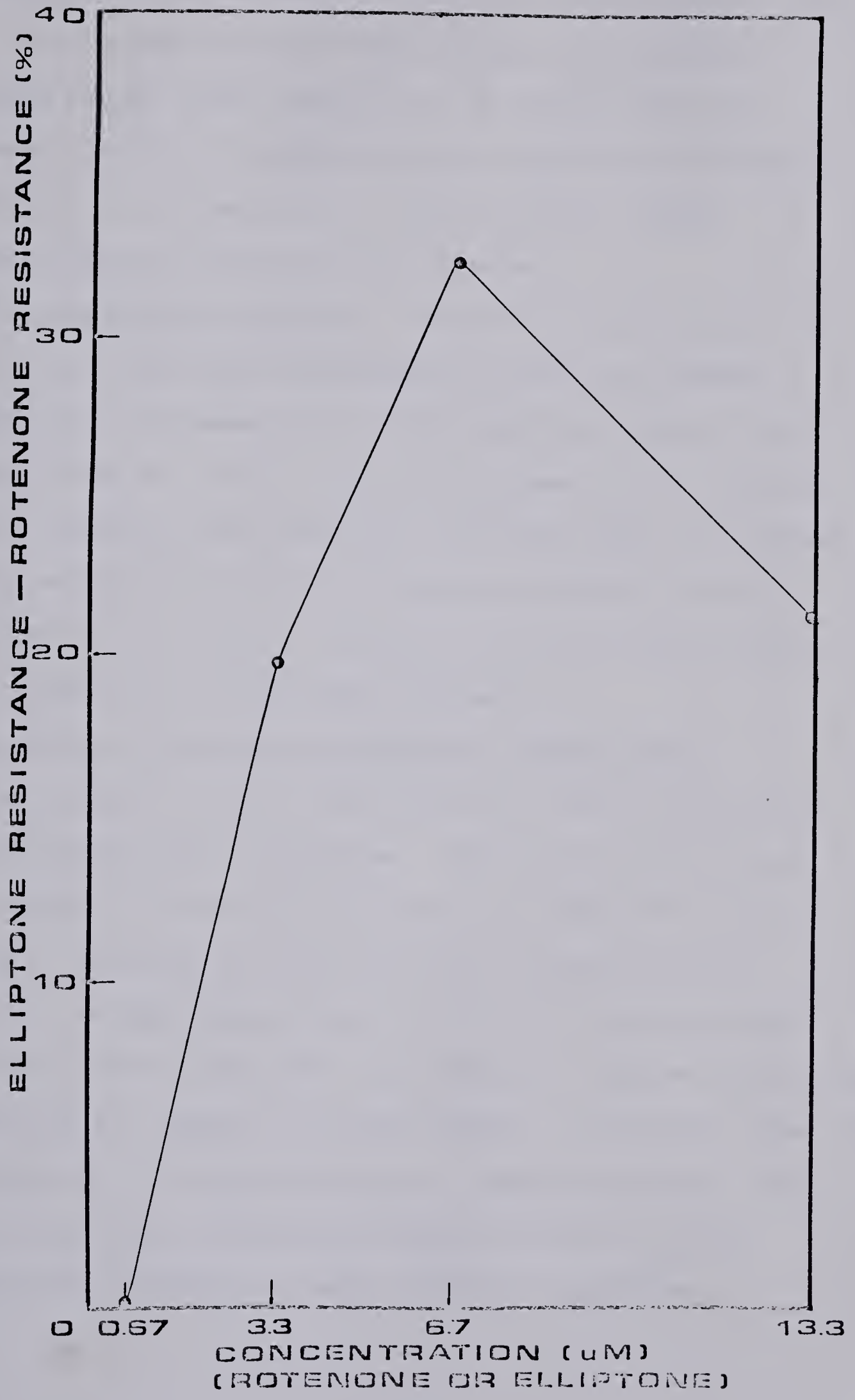

0.1A
260
280
300
320
340
360
380
860
nm

Fig. 8 The difference between elliptone and rotenone resistance in pea cotyledon mitochondrial respiration when malate is the substrate.

Mitochondria from 5 day old cotyledons were isolated and assayed as in Materials and Methods. Each point represents the mean of 3 separate preparations of mitochondria and 2 separate preparations of elliptone. The mean deviation was $\pm$ 7%.

Assay medium: As in Fig. 3.


ELLIPTONE RESISTANCE — ROTENONE RESISTANCE (%)
40
30
20
10
0
0.67
3.3
6.7
13.3
CONCENTRATION (uM)
(ROTENONE OR ELLIPTONE)

Maximum inhibition was reached at elliptone concentrations of 13.3 µM.

Malic enzyme was competitively inhibited by elliptone. Maximum inhibition was caused by 13.3 µM elliptone (Fig. 5). However, the effect of elliptone was not significantly different from that of rotenone ($t(2)\ 5 \leq .69$) (Zar, 1974). Malate dehydrogenase was not affected by elliptone.

Products of Malate Oxidation. The pattern of oxaloacetate production, hence malate dehydrogenase activity, was markedly different in the presence of 13.3 µM rotenone as compared to its absence (Fig. 9:A and B). Malate dehydrogenase activity appeared to be stimulated by the addition of ADP in the absence of rotenone. After ten minutes the pattern of product accumulation was much the same in both samples. Pyruvate production was little affected by the addition of rotenone (Fig. 9:A and B).

The Effect of Rotenone on the Electron Transport Chain.

Spectrophotometric scans of the respiratory pigments were done on isolated mitochondria preparations. Rotenone treatment resulted in reduction of NAD and the cytochrome chain (Table VI). In all cases the reduction was greater than that in the presence of cyanide. No NADH peak was found at 340 nm when cyanide was used. Rotenone treatment resulted in less reduction of NAD and the cytochrome chain than did rotenone and cyanide together. The percent change for NADH was very low because there was an immediate increase in NADH. Both rotenone, and rotenone plus cyanide resulted in a large accumulation of NADH and a small reduction of cytochrome b.

Fig. 9 A. and B. The products of malate oxidation in the presence and absence of rotenone.

Mitochondria from 7 day old pea cotyledons were isolated as described in Materials and Methods. Assays of the reaction products were as in Materials and Methods. Each point represents the mean of 3 separate preparations of mitochondria.*

A) Standard

—●— oxaloacetate —⊖— pyruvate

Assay medium: As in Materials and Methods.

* The mean deviation was ± 0.09 µmoles/mg protein

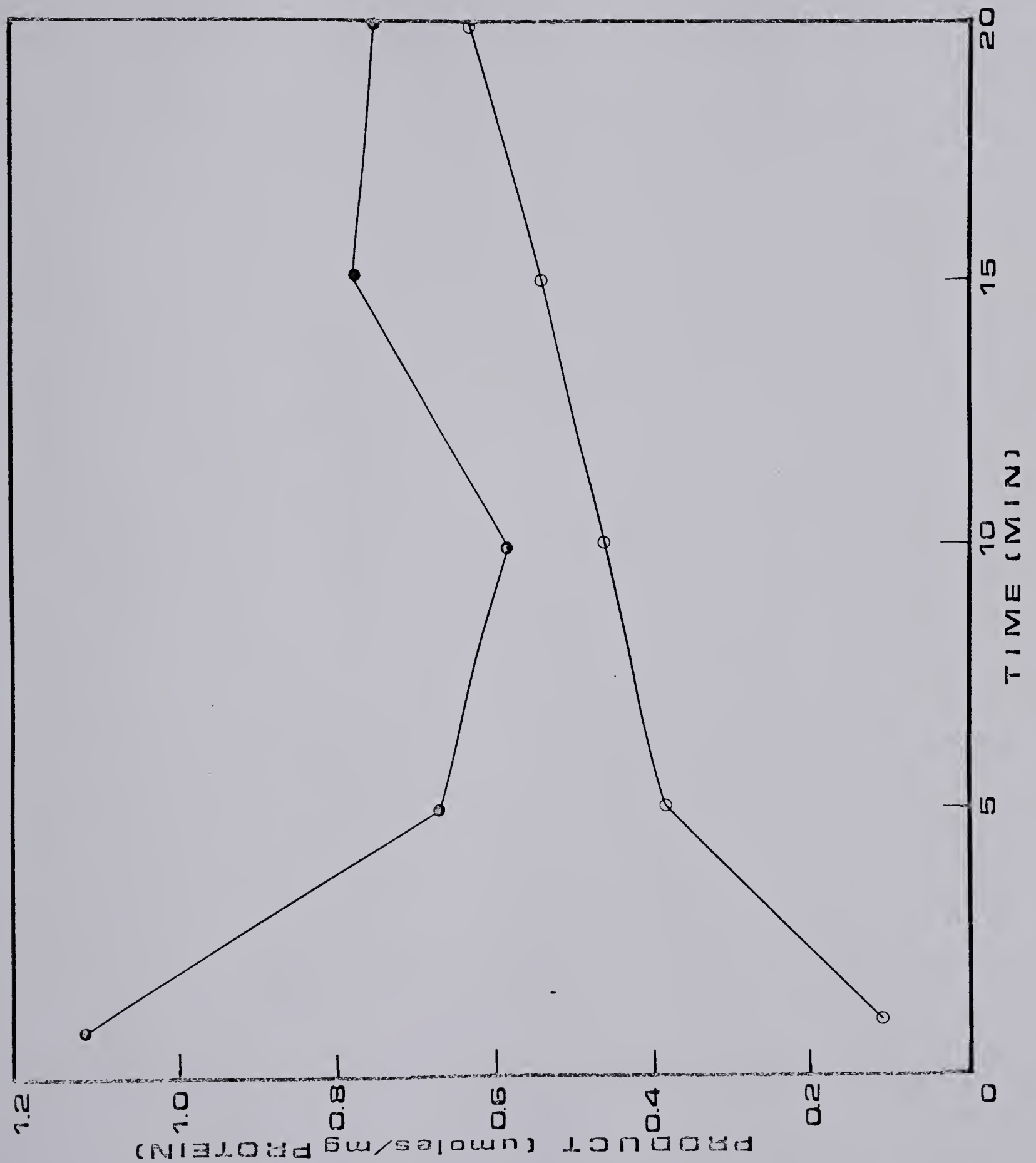

PRODUCT (umoles/mg PROTEIN)
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
TIME (MIN)

Fig. 9: B. 13.3 μM rotenone

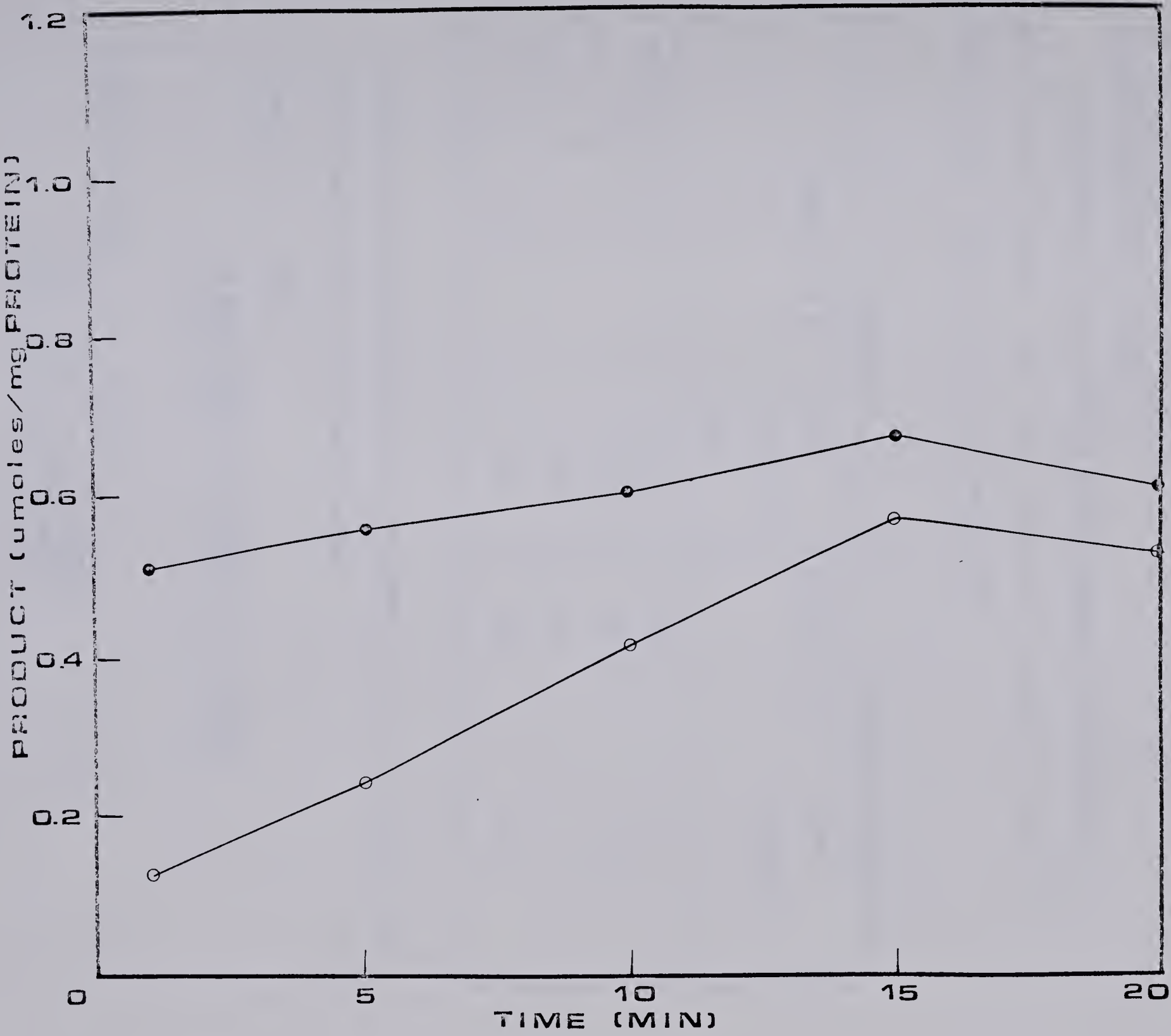

PRODUCT (umoles/mg PROTEIN)
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
TIME (MIN)

Table VI. Reduction of NAD and the Cytochrome Chain in Pea Cotyledon Mitochondria Oxidizing Malate in the Presence of Rotenone or of Rotenone Plus Cyanide.

Mitochondria from 6 day old pea cotyledons were isolated as in Materials and Methods. Each value represents the mean of 3 separate preparations. The mean deviation was ± 10%

	Rotenone		Rotenone and Cyanide	
	Change (%)[a]	Final Absorbance (%)[b]	Change (%)[a]	Final Absorbance (%)[b]
NADH	12	300	417	400
cyt b	126	230	329	280
cyt c	55	50	149	60
cyt aa_3	122	180	335	230

γ peaks were compared when 13.3 μM rotenone or 13.3 μM rotenone plus 5 mM KCN were added to the assay medium.

[a]Change (%) equals:

$$\frac{\Delta \text{ A from time 0 to 15 min} \quad \text{X 100}}{\Delta \text{ A from time 0 to 15 min for KCN sample}}$$

[b]Final Absorbance (%) equals:

$$\frac{\text{A at 15 min} \quad \text{X 100}}{\text{A at 15 min for KCN sample}}$$

DISCUSSION

Preparative Methods. Many studies of mitochondrial respiratory activity are done with mitochondria isolated by simple differential centrifugation. Previous studies of mitochondrial purity and intactness have shown that this method of preparation does not remove microsomal contamination nor does it remove contaminating proteins and broken mitochondria (Douce et al, 1972; James and Spencer, 1979; Solomos et al, 1972). The presence of lipoxygenase on the outer membrane of mitochondria prepared by simple differential centrifugation has been noted (Siedow and Girvin, 1980). The use of this method for preparation of mitochondria could lead to erroneous results in the present inhibitor studies.

The reduction of exogenous cytochrome c by succinate has been used as a method to determine the "intactness" of mitochondria (Douce et al, 1972; Palmer, 1976). The presence of antimycin A-resistant NADPH-cytochrome c reductase indicates microsomal contamination (Palmer, 1976; James and Spencer, 1979). Both preparations contained little broken mitochondria and were relatively free from microsomal contamination. The density gradient preparations were better in these respects. Microsomes decrease rotenone reactivity because of hydroxylation at the 12 α position of the B/C ring juncture and at 6', 7' and 8' of the isopropenyl group (Yamamoto et al, 1971). The high rate of rotenone-resistant respiration in mitochondria prepared by simple differential centrifugation compared to those prepared by the density gradient method was probably caused by microsomal contamination (Table I).

The Effects of Rotenone on α-Ketoglutarate and Malate Oxidation. Mitochondria oxidizing α-ketoglutarate exhibited very high rates of rotenone resistance (Table II and Fig. 2). Other workers (Marx and Brinkmann, 1978) have found the percent resistance to be around 60 to 66% in mitochondria isolated from 9 to 12 day old broad bean epicotyls. Resistance of α-ketoglutarate oxidation is therefore approximatetly two times the resistance obtained for malate oxidation (Fig. 3: A and B). The pattern of rotenone resistance as age increases was different for malate and α-ketoglutarate oxidation.

Inhibition of malate oxidation closely followed the respiratory control ratio (Fig. 4). This suggests that the "fitness" of the mitochondria determines the extent to which inhibition occurs. From the onset of inhibition to four days there is an increase in structural development of the mitochondria. This is paralleled by an increase in respiration rates and phosphorylation (Malhotra and Spencer, 1973). Although maximum oxygen uptake does not occur until day six, respiratory activity is already decreasing (Malhotra and Spencer, 1973). Succinate dehydrogenase and cytochrome oxidase activity drops quite rapidly from five days to seven days (Solomos et al, 1972). Therefore, the structural development of the mitochondria would be expected to control the "fitness" and therefore the resistance to rotenone up to four days. Thereafter, the respiration rates and rates of oxygen uptake probably alter the response to rotenone. This is much different from the development of the cyanide resistant pathway where resistance peaks at about six days. The initial rates of cyanide resistance are very low and do not rise above 30% until the fourth day (James and Spencer, 1979).

The low percent resistance of mitochondria isolated from four day old cotyledons probably reflects the high rate of State 3 respiration. The actual rate of oxygen consumption in the presence of varying concentrations of rotenone in four day old mitochondria was only slightly lower than that for five day old mitochondria. However, the State 3 rate of oxygen uptake prior to inhibition was much greater in the four day old mitochondria than in the five day old mitochondria (results not shown). The decrease in ADP:O ratios caused by rotenone supports other work showing that the first site of phosphorylation is bypassed when mitochondria are oxidizing NAD-linked substrates.

Work on the development of mitochondria (Bain and Mercer, 1956;1965) has shown that the development of the embryonic axis controls the activation of the electron transport chain. This is referred to as stage one (Bain and Mercer, 1956; 1965), and occurs from zero to two days in pea cotyledon mitochondria (James and Spencer, 1979).

Stage three occurs from five days and is a period of mobilization of reserves within the cotyledons (Bain and Mercer, 1956; 1965; James and Spencer, 1979). At this time respiration rates are high as is rotenone resistance when malate is the substrate (Fig. 3:A and B). This is consistent with the hypothesis put forth by Coleman and Palmer (1972) that the rotenone resistant pathway functions to control glycolysis by controlling the NAD/NADH ratio, as well as functioning in the production of organic skeletons.

As Wiskich and Day noted (1979), the recovered oxygen uptake rate, after treatment with piericidin A, observed by Palmer and

Arron (1976) with malate as the substrate was equivalent to that for α-ketoglutarate or citrate oxygen uptake in the presence of piericidin A in mitochondria isolated from Jerusalem artichoke tubers.

In pea cotyledon mitochondria, both malate and α-ketoglutarate oxidation recovered to approximately the same degree after the initial inhibition by rotenone (Table III). Again, the recovered rate of malate oxidation was much the same as the inhibited rate of α-ketoglutarate oxidation. This is consistent with the idea that there is at least two sites of rotenone inhibition. One that is permanent and affects all NAD-linked substrates — probably the NADH dehydrogenase; and the other that is specific for malate oxidation and is a transient inhibition — probably malic enzyme or malate dehydrogenase.

In cauliflower bud mitochondria this transient inhibition could be alleviated if an oxaloacetate removal system was present (Wiskich and Day, 1979). Other workers have found this to be unnecessary in uninhibited mitochondria (Brunton and Palmer, 1973; Coleman and Palmer, 1972), whereas Day and Wiskich found this to be a necessary precaution (1974a; 1979). Therefore the buildup of oxaloacetate may be a problem peculiar to their system. However, if this is not the case malate dehydrogenase activity may be inhibited or reversed as there is also an accumulation of NADH (Day et al, 1976; La Noue, Bryla and Williamson, 1972), thus resulting in the rapid transient inhibition. However, oxaloacetate has been found to have little effect on malate oxidation, while inhibiting pyruvate and citrate oxidation (Brunton and

Palmer, 1973; Coleman and Palmer, 1972). Other workers have found oxaloacetate to have identical effects on all NAD-linked substrates (Douce and Bonner, 1972). As rotenone causes two different effects depending upon the substrate, this suggests that rotenone inhibition does not involve oxaloacetate accumulation. From the study of reaction products this definitely is the case (Fig. 9: A and B). The most plausible explanation for the loss of the transient inhibition would be that malate oxidation is switched from one enzyme to the other.

Localization of the "Rotenone-Resistant Pathway." SHAM and cyanide were added in conjuction with rotenone to determine the path of electron flow from the rotenone block to the terminal oxidase (Table V). SHAM is an inhibitor of the cyanide-resistant pathway and cyanide is an inhibitor of the phosphorylating pathway. The inhibitor of the pathway that the electrons flow to from the rotenone-resistant pathway should therefore reduce the electron flow to zero when used in conjuction with rotenone. Rustin and Moreau (1979) found the rotenone-resistant pathway to be linked to the cyanide-resistant pathway. They found that SHAM inhibited rotenone-resistant respiration in mitochondria isolated from cauliflower buds. However, NAD rather than rotenone was used to activate the rotenone-resistant pathway. In contrast to this, pea cotyledon mitochondria oxidizing malate in the presence of rotenone were inhibited by cyanide and were unaffected by SHAM (Table 1V). The 37% resistance to cyanide is approximately the resistant rate noted by others (James and Spencer, 1979). Rotenone and cyanide together reduced the resistance to 10%. This is probably a result of reversed electron flow or electrons

trickling by the block, possibly by attachment and detachment of rotenone.

Enzyme Inhibition. Although many workers have postulated from work with whole mitochondria that malic enzyme is associated with the rotenone-resistant pathway and malate dehydrogenase is associated with the rotenone-sensitive pathway (Brunton and Palmer, 1973; Coleman and Palmer, 1972; Palmer et al, 1978) to date the purified enzymes have not been studied. Contrary to this view, isolated pea mitochondrial malic enzyme was competitively inhibited by rotenone when NAD was the cofactor (Fig. 5) and malate dehydrogenase was unaffected when NADH was the cofactor (Table V).

The pH at which mitochondria are isolated and assayed may affect the activity of these enzymes. Most workers isolate and assay at pH 7.2 to 7.4. The pH optimum for malic enzyme is between 6.7 and 6.9 (Arron and Edwards, 1980). Activity drops off quickly above this pH, therefore the purification and assay of malic enzyme was done at pH 6.8, while the isolation and assay of intact mitochondria was at pH 7.1. Although the inner mitochondrial membrane acts as an insulating barrier to protons, an increase in the external pH results in an increase in the matrix pH (Neuburger and Douce, in press). In addition to this, at alkaline pHs bicarbonate inhibits malic enzyme (Neuburger and Douce, in press). Therefore if the pH is too high the reaction products (the major method for determining which enzyme is functioning), would not reflect the true effect of rotenone.

The Deactivation of Rotenone. As rotenone inhibition is transient, it may be deactivated within the mitochondrion. This appears to be the case, as elliptone was recovered from mitochondria respiring

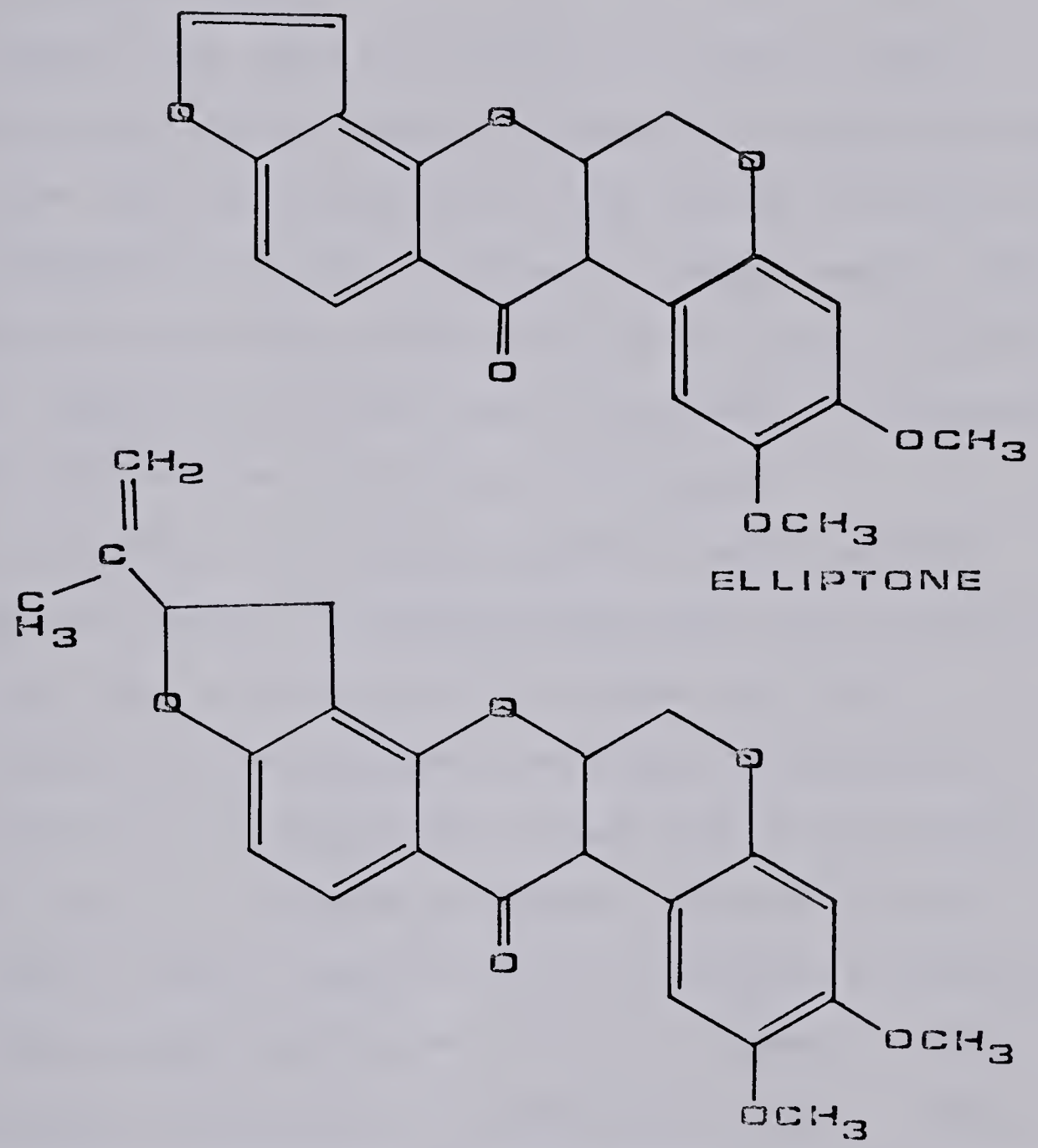
in the presence of rotenone in concentrations comparable to the concentration of rotenone added (Fig. 6 and Fig. 7). Elliptone differs from rotenone by the removal of the isopropenyl group on the tetrahydrofuran ring, resulting in the unsaturation of the ring (Fig. 10). Yamamoto et al (1971) suggested that the deactivation could be non-metabolic. My work confirmed this, as enzymically inactive mitochondria were able to deactivate rotenone.

Elliptone was not as strong an inhibitor as rotenone with intact mitochondria (Fig. 8). The outer mitochondrial membrane would be freely permeable to rotenone and elliptone, however permeability of the inner membrane is doubtful. The effect of rotenone on intact mitochondria therefore may be to distort the membrane because of attachment to lipid in the membrane thus disrupting electron transport. As the lipid environment is important in determining the degree of inhibition (Gutman et al, 1970) and isopropenyl groups increase the molecules' liposolubility (Nath, Venkitasubramanian and Krishnamurti, 1980), this would explain the decreased inhibition caused by elliptone. Further to this, the insecticidal activity of the rotenoids is decreased by the removal of alkyl groups (Muraska and Terada, 1972).

Elliptone causes inhibition of malic enzyme to the same degree as does rotenone (Fig. 5). This is consistent with an effect within the membrane in addition to malic enzyme inhibition.

Products of Malate Oxidation. Pyruvate has been found to be the only product of malate oxidation in mitochondria inhibited by rotenone (Coleman and Palmer, 1972; Palmer and Arron, 1976; Palmer et al, 1978). However, Palmer and his co-workers included

Fig. 10 The chemical structures of rotenone and elliptone.


OCH3
OCH3
ELLIPTONE
CH2
C
CH3
OCH3
OCH3
ROTENONE

NAD in their reaction medium. It has been shown that added NAD stimulates malate oxidation via malate dehydrogenase in species that have low endogenous NAD (Neuburger and Douce, 1978). Therefore the true rotenone inhibition could be masked when NAD is added to the reaction medium.

Another factor which could affect the products of malate oxidation is the initial substrate concentration. Some workers have found that malate dehydrogenase has a high affinity for malate and malic enzyme has a low affinity for malate (Wedding and Pap, 1976), whereas others have found the opposite to be true (Brunton and Palmer, 1973). Therefore, it is best to use a high substrate concentration, as this will maximize the activity of both enzymes.

The accumulation of reaction products of malate oxidation did not fully support the purified enzyme results (Fig. 9:A and B). The final levels of oxaloacetate and pyruvate were lower in the inhibited mitochondria than in the uninhibited mitochondria. The initial level of oxaloacetate was much lower in the rotenone-treated than in the untreated mitochondria. However, the very high initial level of oxaloacetate in the uninhibited mitochondria may inhibit malate dehydrogenase as there is a dramatic decrease in the level of oxaloacetate. The apparently low level of malic enzyme inhibition in vitro may be caused by interactions between malate dehydrogenase and malic enzyme. They would both be expected to utilize the same substrate and cofactor pool, in addition to requiring the other product (oxaloacetate or pyruvate) for product removal via the citrate condensing enzyme.

The Effect of Rotenone on the Electron Transport Chain. The accumulation of NADH in mitochondria inhibited by rotenone has been noted (LaNoue et al, 1972; Oberg, 1961; Ragan and Garland, 1971). Measurements of NADH show that NADH does accumulate (Table VI). The reduction is greatest when rotenone and cyanide are used together.

The entire cytochrome chain is reduced in the presence of rotenone. Again this reduction is greatest when rotenone and cyanide are used together. This can be explained by the previous inhibitor studies. As approximately 35% of the electrons flow through the cyanide-resistant pathway and this pathway is not accessible from the rotenone-resistant pathway there would be greater reduction in the cyanide-sensitive pathway. The addition of cyanide would then further increase the reduction. From this it appears that a rotenone-resistant pathway exists for the transport of electrons from the NAD-linked substrates to cytochrome b. Malic enzyme is sensitive to rotenone. However, NADH dehydrogenases are also involved as there is a large excess of NADH. This suggests there is compartmentalization within the matrix, either physical or organizational, as the NAD available to malate dehydrogenase is not available to malic enzyme. Malate dehydrogenase does not appear to be linked to an NADH dehydrogenase that is linked to phosphorylation. This would then make malic enzyme the main enzyme for the decarboxylation of malate and would therefore support Palmer's modified Krebs cycle (Palmer, 1976).

From this work and the work of others a scheme for rotenone resistance was devised (Fig. 11). Malic enzyme functions to remove oxaloacetate produced from malate dehydrogenase. Most

Fig. 11: A, B & C. Proposed pathways for the oxidation of NAD-linked sustrates in mitochondria isolated from etiolated pea cotyledons.

A. Uninhibited oxidation of NAD-linked substrates.

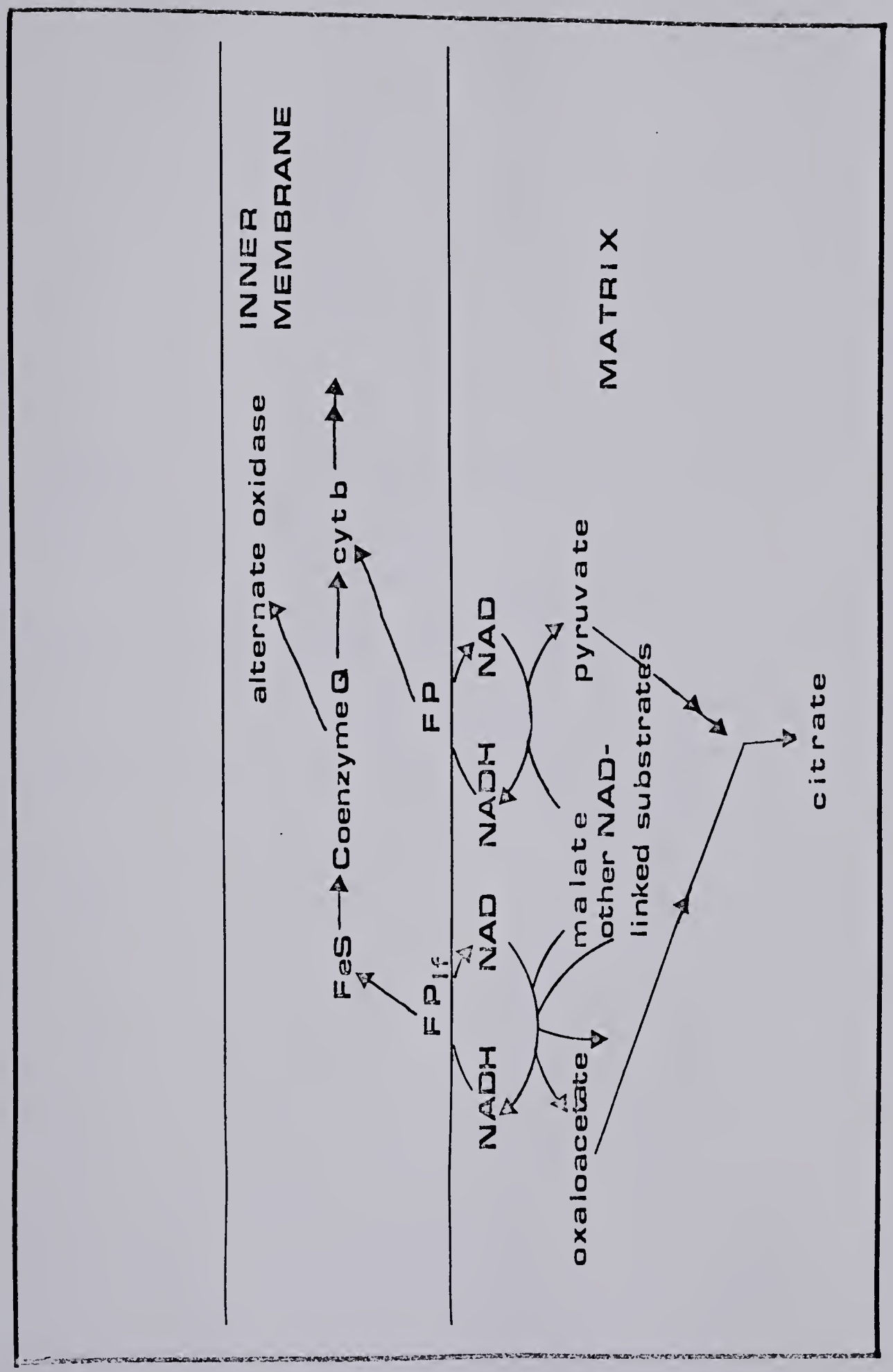

INNER
MEMBRANE
MATRIX
alternate oxidase
CoenzymeQ
cyt b
FP
NAD
pyruvate
NADH
NAD-
linked substrates
citrate
FeS
FP
NAD
NADH
malate
other NAD-
oxaloacetate

Fig. 11: A, B & C. Proposed pathways for the oxidation of NAD-linked substrates in mitochondria isolated from pea cotyledons.

B. Initial inhibition of the oxidation of NAD-linked substrates by rotenone.

■ and the absence of arrows denotes rotenone inhibition.

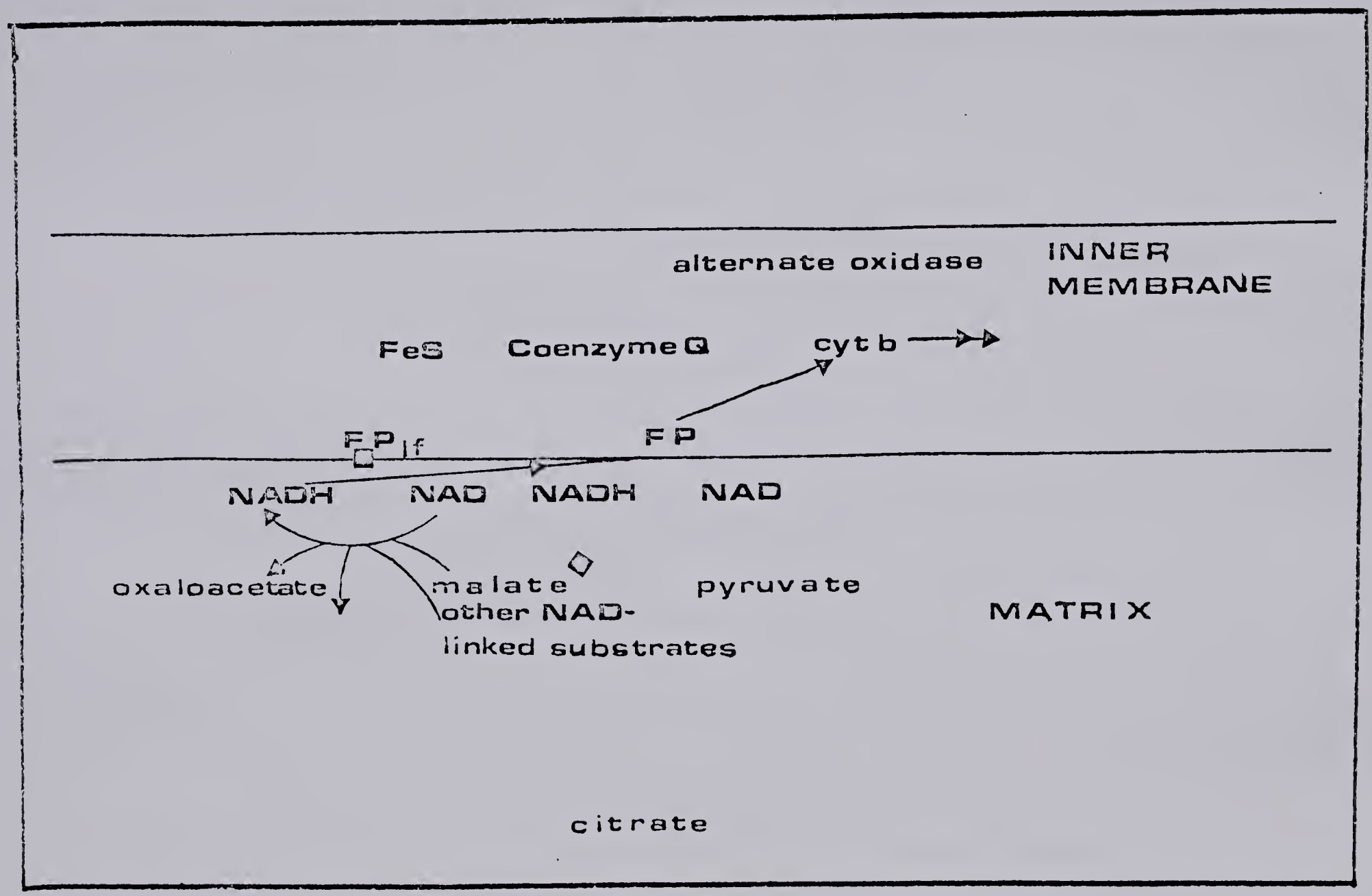
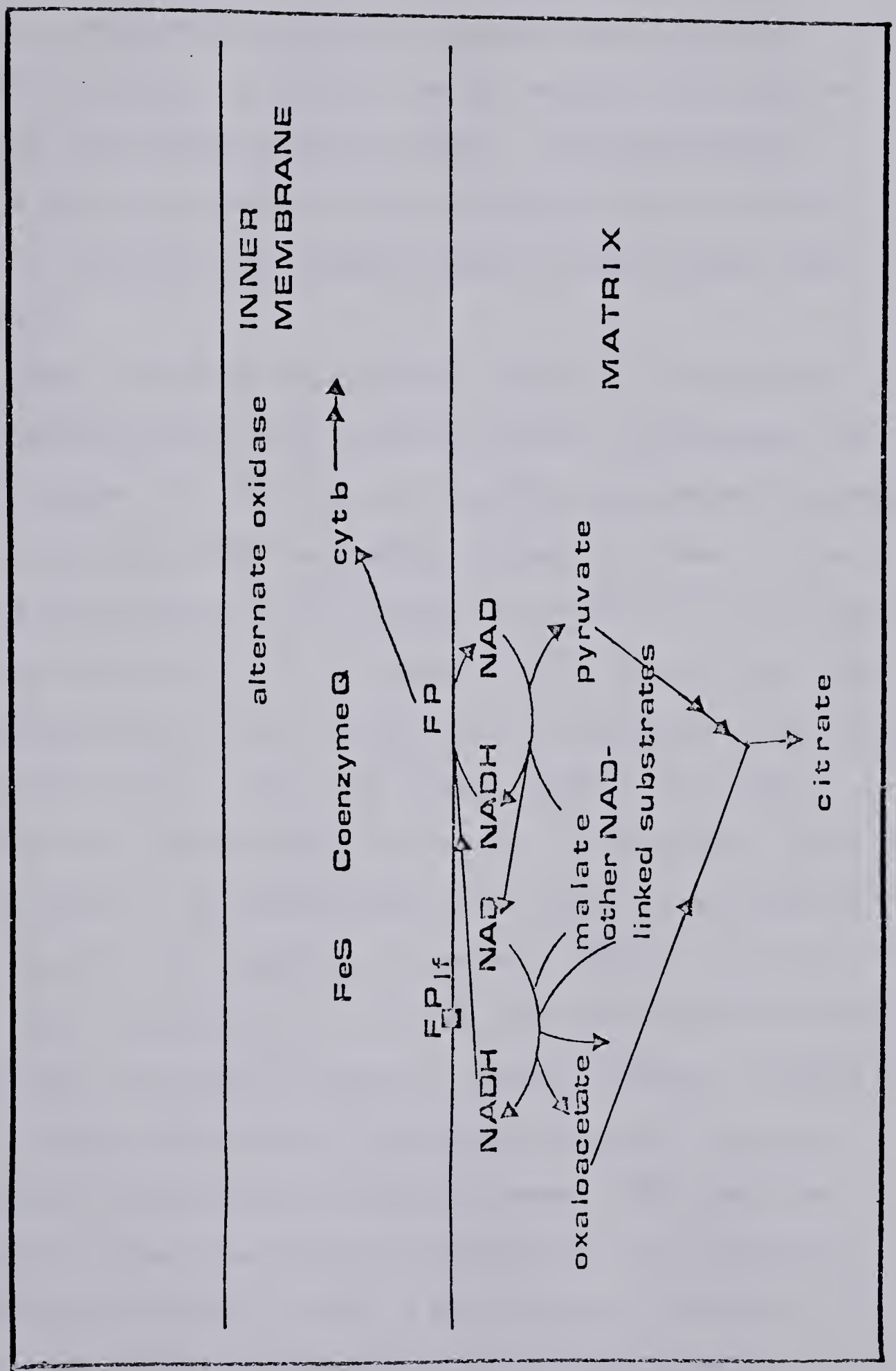

alternate oxidase
INNER MEMBRANE
FeS
Coenzyme Q
cyt b
FP
FP lf
NADH
NAD
NADH
NAD
oxaloacetate
malate
other NAD-linked substrates
pyruvate
MATRIX
citrate

Fig. 11: A, B & C. Proposed pathways for the oxidation of NAD-linked substrates in mitochondria isolated from pea cotyledons.

C. Permanent inhibition of the oxidation of NAD-linked substrates by rotenone.

■ and the absence of arrows denotes rotenone inhibition.


INNER
MEMBRANE
MATRIX
alternate oxidase
cyt b
Coenzyme Q
FeS
FP
FP1
NADH
NAD
NADH
NAD
malate
other NAD-
linked substrates
oxaloacetate
pyruvate
citrate

of the electrons will preferentially enter the electron transport chain at Fp_{1f}. This could be controlled by variations in redox potentials between the two flavoproteins or by the electron transport capacity of the two pathways. According to work on the cyanide-resistant pathway, approximately 65% of the electrons utilize the sensitive pathway (James and Spencer, 1979). Therefore fewer electrons would be able to enter this pathway.

When rotenone is added there is inhibition at two sites, the NADH dehydrogenase associated with malate dehydrogenase and malic enzyme. The inhibition of the NADH dehydrogenase would result in an increase in NADH as noted by a number of workers. There would be approximately 35% reduction in electron flow and therefore oxygen consumption, with a decrease in ADP:O ratios of one. The rotenone probably exerts the inhibition by attaching to the membrane and distorting it. This would cause rotenone to be passively changed into elliptone upon its removal. If elliptone is added to mitochondria it would not be expected to attach to the membrane and therefore there would be approximately 35% less inhibition.

Thus inhibition due to rotenone would affect all NAD-linked substrates and would be permanent. Further evidence to support this includes: SMP electron transport is inhibited 29% in the presence of rotenone when oxidizing exogenous NAD; NAD-linked substrates other than malate are inhibited by 35%; and malate oxidation is inhibited by 30% in the presence of elliptone.

Malic enzyme is competitively inhibited by rotenone, therefore, while the NADH-dehydrogenase is inhibited, there

is also a buildup of oxaloacetate. The oxaloacetate then inhibits the forward reaction of malate dehydrogenase. This would cause a buildup of malate, while at the same time NAD would be expected to be transported into the matrix. These two occurences would allow malic enzyme to produce pyruvate at an ever increasing rate, until the transient inhibition is totally alleviated. At this time malate dehydrogenase would no longer be inhibited. Evidence to support this includes: the pattern of oxaloacetate accumulation in the presence of rotenone parallels the pattern of pyruvate production; and oxaloacetate would increase the rotenone effect on mitochondrial respiration and glutamate would decrease the rotenone effect on mitochondrial respiration (Day and Wiskich, 1974a).

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